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Nuclear PLCβ$_1$ acts as a negative regulator of p45/NF-E2 expression levels in Friend erythroleukemia cells

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Abstract

It is well established that phospholipase C (PLC) β$_1$ plays a role in the nuclear compartment and is involved in the signalling pathway that controls the switching of the erythroleukemia cells programming from an undifferentiated to a differentiated state. Constitutive overexpression of nuclear PLCβ$_1$ has been previously shown to inhibit Friend cells differentiation. For further characterization, we investigated the localization of PLCβ$_1$a and PLCβ$_1$b in Friend cells by fusing their cDNA to enhanced green fluorescent protein (GFP). To investigate the potential target of nuclear PLCβ$_1$ in Friend differentiation, we studied the expression of p45/NF-E2 transcription factor, which is an enhancer binding protein for expression of the β-globin gene and the expression of GATA proteins that are important for the survival and differentiation of erythroid cells. Our data suggest that the overexpression of PLCβ$_1$ (both 1a and 1b) only in the nuclear compartment significantly reduces the expression of p45/NF-E2. The effect observed is attributable to the specific action of nuclear PLCβ$_1$ signalling given that GATA-1 and GATA-3 are not affected at all. Here we show the existence of a unique target, i.e. the transcription factor p45/NF-E2, whose expression is specifically inhibited by the nuclear signalling evoked by PLCβ$_1$ forms.

Keywords: Phospholipase Cβ$_1$; Nucleus; NF-E2; Erythroleukemia; Inositol lipid; GATA family

1. Introduction

Murine erythroleukemia cells are widely used as a model of erythroid differentiation and provide a useful system for studying the mechanism that controls globin gene expression. These Friend virus-transformed cells can be induced to differentiate into erythroid cells by the addition of DMSO[1] or other chemical agents. The cells begin to synthesize a variety of erythroid-specific proteins, including β-globin. The existence of a signal transduction system in the nucleus, based on polyphosphoinositide breakdown mediated by specific phospholipase C (PLC), has been widely documented [1-4].

It has been reported previously, by us and others, that the erythroid differentiation of murine erythroleukemia cells is associated with a down-regulation of the nuclear PLCβ$_1$ [5]. PLCβ$_1$ is composed of two subtypes, 150 kDa PLCβ$_1$a and 140 kDa PLCβ$_1$b [6], which are detectable in both cytosolic and nuclear fraction even if PLCβ$_1$b is located almost exclusively in the nucleus [7]. In a previous study, which we conducted to elucidate the functional role of nuclear PLCβ$_1$ in the regulation of growth and differentiation of Friend erythroleukemia cells, we express PLCβ$_1$ in the nucleus and we have demonstrated that this overexpression of the PLCβ$_1$ was sufficient to block erythroid differentiation of Friend cells induced by DMSO [8].

The program of biochemical and molecular events necessary for the commitment of erythroid cell differentiation is particularly well characterized in murine Friend erythroleukemia cell line. When exposed to DMSO or other inducers, Friend cells undergo phenotypic changes that resemble the final stage of normal erythropoiesis. The most striking feature is the increased synthesis of globin chains, which

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results primarily from the activation of globin gene transcription. Expression of globin gene is dependent on far-upstream locus control region [9]. The promoter of globin genes contains target sites for NF-E2 and GATA transcription factors. In Friend erythroleukemia cells, DMSO increases the activity of NF-E2 that is an obligate heterodimer composed of two different subunits, p45, which is restricted to hematopoietic cells, and an ubiquitously expressed p18 [10,11]. p45/NF-E2 contains a basic region leucin zipper DNA binding domain and it plays a critical role in erythroid differentiation as an enhancer-binding protein for expression of β-globin gene. The promoter and enhancer of globin genes contain also target sites for the transcription factor GATA-1, a transcription factor belonging to the GATA family, essential for various aspects of the development of the hematopoietic lineages [12]. Three members of the GATA family have emerged as important transcription factors in hematopoietic cells. GATA-1, the founding member of this family, is expressed at high level in erythroid cells, mast cells, megakaryocytes, eosinophils and at a low level in multipotential progenitors [13]. GATA-2 is expressed in broad distribution among hematopoietic cells, with particularly prominent expression in early progenitors [14]. GATA-3, which is expressed in T lymphocytes, is essential for T-lymphoid cell development [15]. GATA-1 and p45/NF-E2 appear to be the major cell-restricted, DNA-binding protein that interact directly with the DNA of the locus control region cores.

Therefore, in order to understand which among the transcription factors that controls the transcription of globin gene is the possible target of nuclear PLC signaling activity in erythroid differentiation, we decided to overexpress, in the Friend erythroleukemia cells, the PLCβ$_1$a, PLCβ$_1$b and a mutant in the COOH-terminal region, M2b, lacking the ability of localizing at the nucleus.

Our experiments show that when Friend cells are induced to differentiate along the erythroid lineage, a forced expression of nuclear PLCβ$_1$ blocks specifically the expression of p45/NF-E2, while GATA family is not affected.

2. Materials and methods

2.1. Construction of expression vectors and transfection

The full-length cDNA for rat PLCβ$_1$a [16], the full-length cDNA for rat PLCβ$_1$b [6] and the mutant for nuclear localization sequence, in which by site-directed mutagenesis lysine residues 1056, 1063 and 1070 in region 2 of the COOH-terminus were substituted with isoleucine [17], were cloned into pRc/CMV (Invitrogen) expression vector plasmid as described elsewhere [18]. Friend cells were transfected using the following protocol. Briefly, a mixture of 2.5 μg of plasmid DNA and 10 μl of Transfectam (Promega) was added to 1×10^5 cells in one well of a six-well plate for 24 h The transformants were selected by limiting dilution in medium containing the neomycin analogue G418 at a concentration of 500 ng/ml. Clones were harvested and expanded separately in the presence of G418. The full cDNA of PLCβ$_1$a PLCβ$_1$b and PLCβ$_1$M2b with *Eco*RI sites in the 5′ and 3′ termini was produced by PCR. The PCR products for the PLCβ$_1$s was digest with *Eco*RI and subcloned in the *Eco*RI site of the pEGFP vector (Clontech Labs, Palo Alto, CA), preserving the reading frame.

For experiments with green fluorescent protein (GFP)-construct, transiently transfected Friend cells were plated onto glass coverslip 48 h before observation. Then, the coverslips were mounted on a heated stage with the medium temperature kept at 37 °C. The fluorescence of GFP in living cells was examined in a Zeiss Axiophot fluorescence microscope.

2.2. Cell culture

Murine erythroleukemia cells (Friend cells, clone 707) were grown in RPMI 1640 supplemented with 10% foetal calf serum. Erythroid differentiation was induced by addition of either 1.5% DMSO (v/v) for the indicated times.

2.3. Isolation of nuclei

A hypotonic shock combined with nonionic detergent, essentially described by Martelli et al. [19], has been used. In addition to 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, 1 mM ethylene glycol-bis(aminoethyl ether) tetraacetic acid, 10 μg/ml leupeptin, 0.3 μM aprotinin, 15 μg/ml calpain I inhibitor and 7.5 μg/ml calpain II inhibitor were also added to the buffers. Nuclear purity was assessed by detection of β-tubulin as described in Ref. [20], and only nuclei showing in Western blot a complete absence of β-tubulin were used in the reported experiments.

2.4. Preparation of cytoplasmic fraction

The cytoplasmic fraction was obtained by homogenizing cells with 20 strokes in a Dounce homogeniser 10 mM Tris–Cl, pH 7.8–2 mM $MgCl_2$ plus protease inhibitors as above and then pelleting the nuclei at $400 \times g$. This procedure allows the recovery of pure cytoplasmic fraction and avoid the risk of contamination by nuclear debris that are present in the crude supernatant from nuclear purification.

2.5. Preparation of nuclear extracts

Nuclear extracts were prepared by resuspending the cells in 400 μl of swelling buffer (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl), followed by incubation on ice for 10 min. Next, the lysates were vortexed for 10 s before centrifugation for 1 min at 14,000 rpm at 4 °C. The pelleted nuclei were resuspended in 50 μl of lysis buffer (20 mM Hepes pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) and left on ice for 20 min with occasional mixing.

Subsequently, the lysates were centrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatant was stored at − 70 °C. All buffers were supplemented with 0.5 mM DTT and 0.2 mM PMSF.

2.6. Immunochemical analysis

Immunoblot analysis of 50 μg of nuclear and cytoplasmic fraction were resolved by SDS-PAGE and blotted to nitrocellulose membrane for subsequent immunodetection with the same antibody, followed by detection using ECL (Amersham). For Western blot analysis, proteins from nuclear extract (20 μg) were separated on a polyacrylamide 0.1% SDS gel and transferred on to nitrocellulose paper. The following antibodies were used: polyclonal antibodies to p45/NF-E2, monoclonal antibodies to GATA-1, GATA-2 and GATA-3 from Santa Cruz Biotechnology; polyclonal antibody to PLCβ$_1$ (a and b) from Transduction Laboratory.

3. Results

We sought to compare the distribution of PLCβ$_1$a and PLCβ$_1$b in Friend cells in the presence and absence of DMSO. Fig. 1 shows that PLCβ$_1$a is especially distributed in the cytoplasm while PLCβ$_1$b is, above all, localized in the nucleus. Moreover, both of them are down regulated when Friend erythroleukemia cells, treated with DMSO, differentiate. To investigate the role of PLCβ$_1$ on erythroid differentiation, we stably expressed the wild types and the mutant for the nuclear localization sequence in Friend erythroleukemia cells. The Western blot analysis of the clones overexpressing the PLCβ$_1$a, PLCβ$_1$b and of the mutant PLCβM2b, show that the overexpression of PLCβ$_1$a a gives rise to an equal distribution in both nucleus and cytoplasm. PLCβ$_1$b is essentially nuclear. The overexpression of the mutant M2b, in which lysine residues 1056, 1063 and 1070 were substituted with isoleucine, show that the M2b mutant has lost the capacity for nuclear localization (Fig. 2). In addition, we have to mention that the overexpression of PLCβ$_1$ in the nucleus determines a consistent increase in PLC activity and a resistance to DMSO treatment, in that beta-globin expression is almost completely abolished

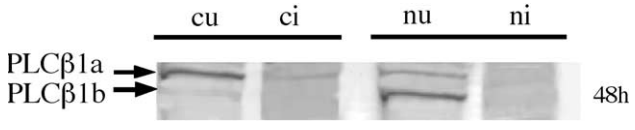

Fig. 1. Intracellular distribution of PLCβ$_1$ phenotype during Friend cells differentiation. Immunochemical analysis with anti-PLCβ$_1$ (a and b). Sixty micrograms of protein loaded for each lane. cu, cytoplasm from uninduced cells; ci, cytoplasm from induced cells; nu, nuclei from uninduced cells; ni, nuclei from induced cells. The intensity of the band of the cytoplasmic fraction of PLCβ$_1$a is stronger than that of the nuclear fraction, while the intensity of the band of the nuclear fraction of PLCβ$_1$b is stronger than that of the cytoplasmic fraction, which is almost completely absent.

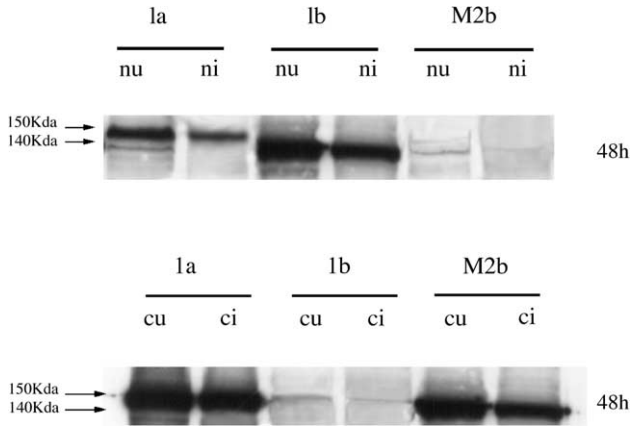

Fig. 2. Analysis of PLCβ$_1$ phenotype in stable transfectants during Friend cells differentiation. nu, ni, cu, ci: as in the Fig. 1. Immunoblot analysis of 60 μg of nuclear lysate of the stable transfectants and immunoblot analysis of 60 μg of cytoplasmic lysate of the stable transfectants. 1a, cells transfected with PLCβ$_1$a; 1b, cells transfected with PLCβ$_1$b; M2b, cells transfected with PLCβ$_1$M2b. The images show both the overexpressed proteins and the endogenous one. The Western blot of stable transfectants reported here are representative of five other clones, which behaved in an identical fashion.

[8]. A further system for monitoring protein localization uses GFP as a molecular tag. Therefore, we investigated the subcellular localization of PLCβ$_1$ subtypes by fusing them to GFP. cDNAs coding for PLCβ$_1$a, PLCβ$_1$b and PLCβM2b were amplified with PCR and introduced into the pEGFP-N1 expression vector, which generated PLCβ$_1$s-EGFP fusion proteins when transfected into mammalian cells. After the cDNA constructs have been verified by sequencing, the GFP-PLCβ$_1$a, b and M2b were transiently overexpressed in Friend cells. As shown in Fig. 3, GFP-PLCβ$_1$b localizes almost exclusively in the cell nucleus. GFP-PLCβ$_1$a accumulates in the cytoplasm, as well as in the nucleus. On the contrary the GFP-PLCβ$_1$M2b mutant distributes throughout specifically the cytoplasm, being completely absent in the nucleus. Therefore, the GFP labeling is in complete agreement with immunochemical data about subcellular localization of PLCβ$_1$ forms.

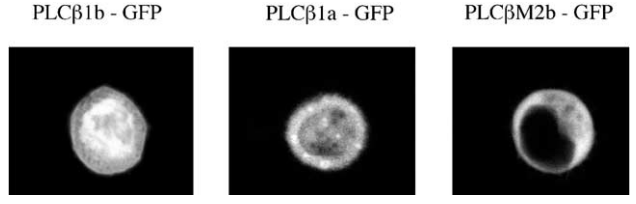

Fig. 3. Intracellular localization of GFP-tagged PLCβ$_1$ subtypes and PLCβ$_1$M2b mutant proteins in living cells. Friend cells growing in medium containing 10% calf serum were transiently transfected with the GFP-tagged constructs of PLCβ$_1$a, PLCβ$_1$b and PLCβ$_1$M2b mutant, respectively. At 48 h post-transfection, cells were viewed with a Zeiss Axiophot fluorescence microscope and photographed. The picture is representative of five separate transfections and all transfected cells showed the same pattern in each experimental condition.

Given that nuclear PLCβ$_1$ is involved in the signaling machinery that controls erythroid differentiation of Friend cells in vitro, we tried to find out the possible targets of nuclear PLCβ1 signaling. Therefore, taking advantage of stable transfectants, with different subcellular localization of PLCβ$_1$, we decided to assess the levels of p45/NF-E2 and GATA proteins by immunoblot analysis. NF-E2 plays a critical role in erythroid differentiation as an enhancer binding-protein for the expression of the β-globin gene and is a differentiative marker in Friend cells differentiation even if the commitment to hemopoietic pathways is not achieved by a single factor, but rather by combinatorial mechanism that take place within 48 h of induction with differentiative agent. Fig. 4 shows the level of expression of the hematopoietic factor p45/NF-E2 in the stable transfectants and in wild type cells cultured for different times (48–72 h) in the presence or absence of DMSO. The overexpression of PLCβ$_1$a and PLCβ$_1$b induces a clear lack of increase in p45/NF-E2 level in the cells treated with DMSO, whereas the overexpression of the mutant PLCβ$_1$M2b did not affect the level of the hematopoietic factor as compared to wild type cells. The time course of p45/NF-E2 expression indicates that this factor, which is absent in undifferentiated erythroid cells [8], is expressed at 48 h after treatment with the inducer and peaks at 72 h when β-globin initiates to be expressed [8].To establish whether p45/NF-E2 was a specific target of nuclear PLCβ$_1$, GATA-1, 2 and 3 expression was also examined in Friend cells induced to differentiate with DMSO since these GATA family transcription factors are important regulators of gene expression in hematopoietic lineages. Fig. 5 shows that GATA-1 and GATA-3 increases up to 72 h in the presence of DMSO. Their expression is the same in wild type cells as well as in clones overexpressing PLCβ$_1$ forms in the nucleus and in the mutant for the nuclear localization

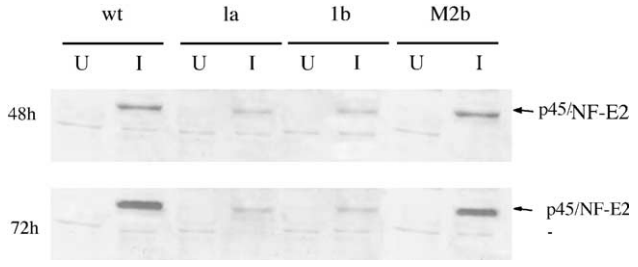

Fig. 4. Enforced expression of the nuclear PLCβ$_1$ is sufficient to block p45/NF-E2. Cells were induced to differentiate for 48 and 72 h with DMSO. Immunoblot analysis of 50 μg of nuclear extracts of wt and stably transfected cells. wt, wild type cells; 1a, cells transfected with PLCβ$_1$a; 1b, cells transfected with PLCβ$_1$b; M2b, cells tranfected with PLCβ1M2b. U, uninduced cells; I, induced cells. The densitometric analysis of the blots indicates that at 48 h, there is a 10-fold increase of the intensity of p45/NF-E2 in induced wt cells, while in PLCβ$_1$a and b clones, the increase is less than 2-fold. At 72 h, the increase in wt cells is more than 20-fold while in PLCβ$_1$a and b clones, the increase is only 2-fold. The Western blot of stable transfectants reported here are representative of five other clones, which behaved exactly as the ones in the figure.

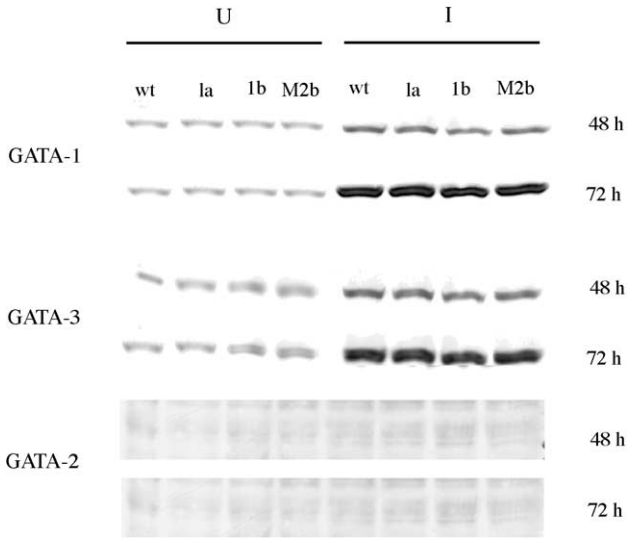

Fig. 5. Effect of PLCβ$_1$ subtypes overexpression on GATA-1, GATA-2 and GATA-3 transcription factors levels. Cells were induced to differentiate for 48 and 72 h with DMSO. Immunoblot analysis of 50 μg of nuclear extracts of wt and stably transfected cells. wt, wild type cells; 1a, cells transfected with PLCb1a; 1b, cells transfected with PLCβ1b; M2b, cells tranfected with PLCβ1M2b. U, uninduced cells; I, induced cells. The Western blot of stable transfectants reported here are representative of five other clones, which behaved in an identical fashion.

sequence, suggesting that GATA-1 and GATA-3 are not affected by the nuclear localization of PLCβ$_1$. Fig. 5 also shows that GATA-2 is not expressed in Friend cells, in agreement with the finding that this transcription factor is highly expressed only in early erythroid cells, which is not the case for Friend cells we dealt with since they do not belong to an early stage of the erythroid lineage [14].

4. Discussion

Friend murine erythroleukemia cells provide a useful system for studying the mechanism that controls both erythroid differentiation and globin gene expression. The aim of this study was to clarify whether the nuclear localization of the PLCβ$_1$ forms is connected to specific targets during differentiation of Friend erythroleukemia cells. It has been demonstrated by our group that PLCβ$_1$ is down regulated when Friend cells treated with DMSO differentiate and synthesize β-globin [20]. Furthermore, it has also been demonstrated that overexpression of a specific, nuclear-localized, PLCβ$_1$ isoform is sufficient to maintain the undifferentiated state of Friend cells induced by DMSO [8], possibly due to a continuous stimulation of the cell cycle. Furthermore, we have recently demonstrated that Friend erythroleukemia cells overexpressing PLCβ$_1$ isoforms that localize to the nucleus (PLCβ$_1$a and b) can grow in the absence of serum because cyclin D3 is also overexpressed, and the active complex phosphorylates pRb, then activating the E2F transcription factor [21].

Previous work showed that nuclear localization depends on a cluster of lysine residues in the COOH-terminal domain [17]. Therefore, we have used the mutant PLCβ_1M2b, lacking the ability of localizing at the nucleus, to find out the possible nuclear target of PLCβ_1 during erythroid differentiation. In this work, we have compared the expression and localization of PLCβ_1 forms in control cells with cells undergoing differentiation that has been induced by DMSO. To do this, we have made use of the GFP technology, which provides unprecedented opportunities to study the enzymes involved in nuclear lipid signalling pathway in living cells [22] and circumvents problems such as contamination by residues of the plasma membrane, antibody cross reaction or fixation and inadequate antibody penetration in the case of fluorescent signal in immunocytochemical investigations. Therefore, to facilitate the detection of transfected cells, the PLCβ subtypes and the mutant for the nuclear localization sequence PLCβM2b were fused to enhanced GFP. The experiments described here demonstrated that the expression of transcription factor p45/NF-E2, a prerequisite in the erythroid differentiation of Friend cells, is modulated by nuclear PLCβ_1, as the overexpression of the nuclear subtypes, instead of the mutant PLCβM2b, abolishes almost completely DMSO-induced p45/NF-E2 expression. It is likely that, in these cells, the overexpression of the mutant for the nuclear localization sequence, PLCβ_1M2b, prevents nuclear PLCβ_1a and b from translocating to the nuclear compartment. This result assigns to nuclear PLCβ_1 a role as a negative regulator of erythroid differentiation and its nuclear signalling might have a relevance for the pathogenesis of cancer since Friend cells with high levels of nuclear wild type PLCβ_1 actively synthesizes DNA even after having been cultured for 3–4 days in the presence of DMSO [8]. We found that expression of GATA-1 was not reduced in Friend cells after nuclear PLCβ_1 overexpression, suggesting that this nuclear signal has indeed a specific target, which is p45/NF-E2, while GATA-1 expression is not affected at all by nuclear PLCβ_1. This implies that there are two different signalling pathways that control the expression levels of these two transcription factors. In fact, a recent report by Bavelloni et al. [23] demonstrates that the binding of the transcription factors GATA-1 to its DNA-binding site is modulated by PI3-Kinase pathway, as wortmannin abolishes almost completely DMSO-induced GATA-1 binding to DNA. On the other hand, it should be remembered that Martelli et al. [24] have shown that nuclear PLCβ_1 strictly depends on its phosphorylation, which is mediated through the MAPK pathway. Furthermore, we have recently demonstrated that there is a cross-talk between the MAPK cascade and a PLC signalling pathway, that is that ERK evoked phosphorylation of PLCβ_1 at serine 982 [25]. Therefore, our results are in agreement with the recent finding that DNA binding and transactivation activity of p45/NF-E2 are regulated by serine/threonine phosphorylation such as a MAPK signaling cascade [11]. It has recently been described that loss of p45/NF-E2 expression is required for the establishment of permanent erythroleukemic cell in culture. The absence of p45/NF-E2 in erythroleukemic cells promotes tumor growth by accelerating the rate of cellular proliferation. This transcription factor has a direct role in the regulation of globin gene expression in erythroid cells, possibly because it functions as a tumor suppressor gene in Friend virus-induced erythroleukemias [26]. The data presented here clearly demonstrated that specific subcellular localization of the two subtypes is responsible for differences in their biological function. Upon DMSO treatment, the two different subtypes PLCβ_1a a and b, when localized in the nucleus, have the same nuclear target, which is the transcription factor p45/NF-E2. This target is highly specific since other transcription factors involved in erythroid differentiation of Friend cells, such as members of GATA family, do not respond to nuclear PLCβ_1 signaling. Therefore, we could assign a specific role for this signalling pathway in Friend cells programming from a differentiative to a proliferative state.

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