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Mass Spectrometry-Based Identification of Y745 of Vav1 as a Tyrosine Residue Crucial in Maturation of Acute Promyelocytic Leukemia-Derived Cells

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Vav1, whose physiological expression is restricted to hematopoietic system, is one of the signaling proteins up-regulated by all-*trans* retinoic acid (ATRA) in acute promyelocytic leukemia (APL)-derived precursors, in which it promotes the overcoming of the differentiation blockade. High levels of tyrosine phosphorylated Vav1 accumulate in differentiating APL-derived cells, suggesting that one or more Vav1 tyrosine residues are involved in neutrophil differentiation of tumoral promyelocytes. Here, we have found that phosphorylation of Vav1 Y174, that is known to regulate Vav1 activity in mature neutrophils, is up-regulated by ATRA in NB4 cells. Nevertheless, this tyrosine residue does not seem crucial for the agonist-induced phenotypical differentiation of APL-derived cells. Mass spectrometry analysis performed on Vav1 from differentiating NB4 cells allowed to identify the highly conserved Y745 residue as a phosphorylated tyrosine that plays crucial roles in the completion of the maturation program of this cell line. In fact, the overexpression of a mutated form of Vav1, in which Y745 was replaced with a phenylalanine, significantly reduced the ATRA-induced CD11b expression and essentially abrogated the differentiation-related acquisition of the migratory capability. Even though the intracellular signaling involving Vav1 phosphorylated in Y745 is unknown, the identification of a tyrosine residue essential for differentiation of tumoral precursors may constitute the basis to identify new specific targets for differentiation therapy of APL.

Keywords: Vav1 • mass spectrometry • NB4 cells • ganulocytic differentiation • tyrosine phosphorylation

Introduction

Acute promyelocytic leukemia (APL) is the M3 subtype of acute myeloid leukemias, characterized by hyperproliferation of progenitors that are committed to terminal differentiation into granulocytes.[1,2] Despite recent clinical studies using arsenic trioxide, anthracyclines and anti-CD33 monoclonal antibodies,[3,4] all-*trans* retinoic acid (ATRA)-based therapy represents, until today, the standard cure of APL patients.[5] Studies on both APL blasts and APL-derived cell lines have elucidated that the treatment with ATRA promotes the completion of their maturation to neutrophils[1,6,7] throughout a complex network that includes the degradation of the PML/RARα fusion protein and the activation of RARα-mediated gene transcription.[8]

Vav1, whose physiological expression is restricted to hematopoietic system,[9] is one of the signaling proteins up-regulated by ATRA in APL-derived promyelocytes.[10,11] At variance with maturation of normal myeloid precursors, in which it seems to be ineffective,[12] Vav1 plays an important role in the agonist induced differentiation of APL-derived cells. In particular, the down-modulation of Vav1 during ATRA treatment prevents maturation while its overexpression in differentiating cells strengthens the effects of the drug.[11] By means of array profiling obtained with differentiating HL-60 cells, we have demonstrated that the forced reduction of Vav1 during treatment with the agonist reduces the number of ATRA-induced genes.[11] In addition, by means of a proteomic approach on both HL-60 and NB4 cells, we have recently demonstrated that Vav1 plays a role in modulating the expression level of the protein tool by means of which ATRA executes

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the maturation program of tumoral promyelocytes,[13] possibly as part of transcriptional complexes.[14]

The best known function of Vav1 is a tyrosine phosphorylation-regulated guanine nucleotide exchange (GEF) activity for small G proteins.[15] However, due to the presence of diverse domains and of several tyrosine residues inside its structure, Vav1 may also interact with a number of different molecules, in both cytoplasm and nuclear compartments, playing additional roles as an adaptor/regulator protein.[16−18]

In neutrophils, Vav1 plays a role in integrin-mediated adhesion and in regulating chemoattractant-induced NADPH oxidase activity of mature cells.[19] A combined deficiency in Vav1 and Vav3, that accounts for 99% of total Vav protein in neutrophils, results in their defective chemoattractant-induced adhesion and integrin mediated phagocytosis.[20] So far, all the roles for Vav1 in mature neutrophils have been described to depend on its GEF activity, regulated by the phosphorylation of Y174 by members of Syk/Zap70 and Src tyrosine kinase families.[21,22] In particular, Syk is critical for β2 integrin-mediated neutrophil migration *in vitro* and plays a fundamental role in neutrophil recruitment during the inflammatory response *in vivo*.[22]

Since high levels of tyrosine phosphorylated Vav1, with largely unknown functions, accumulate in differentiating APL-derived cells,[11] we have long been interested in elucidating the role of phosphotyrosine residues in the diverse potential activities of Vav1 during the completion of the maturation process of tumoral precursors.

We have previously demonstrated that the prominent tyrosine phosphorylation of Vav1 induced by ATRA in both HL-60 and NB4 cells is only in part dependent on Syk.[13,23] However, the Syk-dependent tyrosine phosphorylation of Vav1 is not involved in the ATRA induced expression of the myeloid differentiation marker CD11b.[13] On the other hand, it results essential for the changes of the nuclear shape that occur during neutrophil-like maturation of APL-derived promyelocytes,[13] possibly by regulating PI 3-K activity and, ultimately, by modulating the cytoskeleton associated phosphoinositide pools.[24]

Extending our previous studies, the present work was aimed to identify tyrosine residue(s) of Vav1 whose phosphorylation is induced by ATRA in order to overcome the differentiation blockade of tumoral promyelocytes.

Materials and Methods

Cell Culture and Differentiation. All the used reagents were from Sigma Chemicals Co. (St. Louis, MO) if not otherwise indicated.

The APL-derived NB4 cell line was obtained from the "German Collection of Microorganisms and Cell Cultures" (Braunschweig, Germany) and was cultured in RPMI 1640 (Gibco Laboratories, Grand Islands, NY) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco Laboratories) in a 94/6% (v/v) air/CO_2 atmosphere. Cell density was maintained between 5×10^5/mL and 1.5×10^6/mL.

For neutrophil-like maturation, cells were treated with 1 μM ATRA and the degree of granulocytic differentiation was evaluated by measuring the expression level of the CD11b myeloid surface antigen by direct staining with a PE-conjugated anti-CD11b-antibody (Immunotech, Coulter Company, Marseille, France), as previously reported.[13] After staining, samples were analyzed using flow cytometry (FACScan, Becton-Dickinson, San José, CA) with Lysis II software (Becton-Dickinson). Data collected from 10 000 cells are presented as mean fluorescence intensity values.

To inhibit Syk-dependent tyrosine phosphorylation of Vav1, cells were treated for 4 days with 1 μg/mL Piceatannol, alone or in combination with 1 μM ATRA, as previously reported.[13]

Protein Fractionation by MicroRotofor Isoelectric Focusing. Pellets of NB4 cells (2.5 mg of proteins) were dissolved in 2.5 mL of isoelectric focusing (IEF) buffer (7 M urea, 2 M thiourea, 5 mM dithiothreitol (DTT), 4% (w/v) Chaps and 2% (v/v) Pharmalyte, pH 3−10), applied to MicroRotofor (Bio-Rad Laboratories, Hercules, CA), and electrophoresed according to the manufacturer's instructions for 3 h at constant power of 1 W at 20 °C. After electrophoresis, protein fractions from each compartment (200 μL) were harvested and an equal volume of 20% (w/v) trichloroacetic acid (TCA) was added. Proteins were recovered by centrifugation at 21 000*g* for 10 min at room temperature. Then, 500 μL of cold acetone was added to the precipitates, and after vortexing and centrifugation at 21 000*g* for 10 min at 4 °C, the supernatant was carefully removed. This washing step was repeated twice to remove TCA. Precipitated proteins were air-dried, dissolved in 30 μL of sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min and subjected to monodimensional electrophoresis. Resolved proteins were stained with Coomassie Brilliant Blue G-250 or transferred to nitrocellulose membranes (Amersham Life Science, Little Chalfont, U.K.).

Preparation of Immunoprecipitates. Vav1 was immunoprecipitated from protein fractions obtained after electrophoresis with MicroRotofor by using an anti-Vav1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), overnight at 4 °C, as previously reported.[25]

Myc-tagged overexpressed Vav1 proteins were immunoprecipitated with an anti-Myc antibody (Santa Cruz Biotechnology) from NB4 whole cell lysates obtained after resuspension of cells in a lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 μg/mL each Aprotinin and Leupeptin, 1 mM Na_3VO_4 and 1 mM PMSF (all from Calbiochem, Merck Chemicals Ltd., Nottingham, U.K.).

In both cases, the immune complexes were precipitated with protein A-Sepharose (Pharmacia, Uppsala, Sweden) and, after washing and resuspension in Laemmli's SDS sample buffer, were subjected to monodimensional electrophoresis followed by immunochemical analysis or by mass spectra analysis.

Immunochemical Analysis. Proteins from lysates and immunoprecipitates were separated on 7.5% (w/v) polyacrylamide denaturing gels and transferred to nitrocellulose membranes (Amersham Life Science).

For analysis of tyrosine phosphorylated proteins, membranes were incubated for 2 h with a monoclonal anti-phosphotyrosine antibody (PY20, Transduction Laboratories, Lexington, KY), as previously described.[13] For analysis of Vav1 phosphorylated on Y174, a specific antibody was used (Santa Cruz Biotechnology), according to the manufacturer's instructions. For analysis of Vav1, the membranes were incubated overnight with the specific polyclonal antibody (Santa Cruz), as previously reported.[13] For the analysis of β-tubulin, the nitrocellulose membranes were reacted with a specific monoclonal antibody (Sigma), as previously reported.[11]

All membranes were then incubated with IgG peroxidase-conjugated secondary antibodies and revealed by chemiluminescence using the ECL system (PerkinElmer, Boston, MA), according to the manufacturer's instructions.

Mass Spectrometry Analysis. The gels containing the native Vav1 immunoprecipitated from MicroRotofor protein fractions and the gels containing the overexpressed wild-type and mutated Vav1 obtained after immunoprecipitation with the anti-Myc antibody were stained with Comassie Brilliant Blue G-250, as previously reported.[11] Bands corresponding to Vav1, identified by their correspondence with proteins used as molecular weight markers, were manually excised using a sterile blade and stored in a solution of 5% (v/v) acetic acid, before being processed for mass spectrometry analysis.

After destaining with 100 mM ammonium bicarbonate (pH 8) and 50% (v/v) acetonitrile (ACN), proteins were reduced and alkylated using solutions of 10 mM DTT and 55 mM iodoacetamide and finally digested with sequencing grade trypsin (Promega, Madison, WI) at 37 °C. After incubation overnight, digestion was stopped by adding trifluoroacetic acid (TFA).

After in-gel digestion, peptides were desalted using C18 ZipTips (Millipore, Bedford, MA) and co-crystallized with a solution of 5 mg/mL α-cyano-4-hydroxycinnamic acid dissolved in ACN and TFA 0.1% (1:1 v/v) on a thin layer of 10 mg/mL α-cyano-4-hydroxycinnamic acid dissolved in ethanol, ACN and TFA 0.1% (v/v) (495:495:10).

Mass spectra were acquired with a Ultraflex III MALDI-TOF/TOF spectrometer (Bruker-Daltonics, Bremen, Germany) operating in positive ion reflection/delayed extraction mode, in mass range 800−4000 Da, after an external calibration with the standard peptide mixture from Bruker-Daltonics (m/z 1046.54, 1296.68, 1347.73, 1619.82, 1758.93, 2093.08, 2465.19, 2932.59, 3494.65). Internal calibration was performed using autolysis peaks from porcine trypsin (m/z 842.509 and 2211.104). Spectra were processed using FlexAnalysis software v3.0 (Bruker Daltonics). Database search was performed using MASCOT_2.2.03 algorithm (www.matrixscience.com) interrogating NCBInr_20080912 database restricted to *Homo sapiens* taxonomy (216 740 sequences) with carbamidomethylation of cysteines as fixed modification, oxidation of methionines and phosphorylation of tyrosines as variable modifications, one missed cleavage site allowed for trypsin and 50 ppm as maximal tolerance. An MS score > 66 indicates identity or extensive homology ($p < 0.05$).

MS/MS spectra were acquired after switching the instrument in the LIFT mode and precursor ions for fragmentation were selected manually. The precursor mass window was set automatically after the precursor ion selection. The FlexAnalysis v3.0 software (Bruker Daltonics) was used for processing the spectra following baseline subtraction, smoothing (Savitsky-Golay) and centroiding.

***In Situ* Mutagenesis.** Mutation of the residue Y745 of Vav1 into phenylalanine was performed using the QuickChange XL II site-directed mutagenesis kit from Stratagene (La Jolla, CA) following the manufacturer's instructions. To perform the mutagenesis reactions to generate Y745F, the following primer pair was used: primer I (5′- GGA GCT GGT GGA GTT TTT CCA GCA GAA CTC TC-3′) and primer II (5′- GAG AGT TCT GCT GGA AAA ACT CCA CCA GCT CC- 3′). Mutation was verified by DNA sequencing.

XL 10-Gold ultracompetent cells (Stratagene) were transformed with the mutagenized vector. To isolate a large quantity of plasmid DNA, the PureYield Plasmid Maxiprep System (Promega) was used according to the protocol provided by the manufacturer.

Overexpression of Vav1. NB4 cells were transiently transfected with a pEF plasmid expressing the human full-length Myc-tagged Vav1 in which the tyrosine 174 was mutated into phenylalanine (Vav1 Y174F, kindly provided by Dr. Weiss, Howard Hughes Medical Institute, San Francisco, CA) or with a plasmid expressing the human full-length Myc-tagged Vav1 in which the tyrosine 745 was mutated into phenylalanine (Vav1 Y745F). To evaluate the efficiency of transfection and to identify the overexpressing cells, the same procedure was performed co-transfecting cells with both pEF Vav-myc and pEGFP plasmids (9:1 ratio). Briefly, 5×10^6 cells were mixed with 10 μg of plasmid DNA resuspended in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) in 400 μL of RPMI 1640 (Gibco Laboratories) plus 20% (v/v) FBS (Gibco Laboratories), then electroporated as previously reported.[11] After 5 h of incubation, cells were resuspended in RPMI 1640 (Gibco Laboratories) plus 10% (v/v) FBS (Gibco Laboratories) at a density of 5×10^5 cells/mL and cultured in control conditions or in the presence of ATRA.

Concerning transfection efficiency, after 24 h from electroporation, the percentage of cells expressing the EGFP protein, evaluated *in vivo* by inverted fluorescence microscopy, ranged from 15 to 25%.

Migration Assay. Cell migration was determined using a 24-Transwell-migration chamber 6.5 mm in diameter (Costar, Boddenheim, Germany) equipped with 8 μm pore size polycarbonate inserts, as previously reported.[26] Briefly, NB4 cells (2×10^5 cells/mL) were resuspended in 100 μL of RPMI 1640 (Gibco Laboratories) plus 2% (v/v) FBS (Gibco Laboratories) and added to the upper compartment of the Transwell inserts. The lower compartment was filled with 600 μL of medium containing 25% (v/v) FBS (Gibco Laboratories). After 6 h of incubation, cells located in the lower compartment were carefully collected and counted.

In experiments with transfected cells, the percentage of green fluorescent cells, overexpressing EGFP, and wild-type or mutated Vav1, in the upper and in the lower compartment, was evaluated by means of flow cytometry (FACScan).

In all cases, the migration rate was expressed as percentage of migrated cells on the total cell number seeded in the upper compartment of the chamber at the beginning of the experiment.

Results

Tyrosine 174 of Vav1 Is Not Crucial for ATRA-Induced Differentiation of NB4 Cells. Immunochemical analysis on Vav1 immunoprecipitated from differentiating NB4 cells demonstrated that, as previously reported, ATRA induces a large increase in amount and tyrosine phosphorylation levels of Vav1. The use of a specific anti-phosphotyrosine antibody on the same immunoprecipitates showed that a significant increase of phosphorylated Vav1 Y174 is induced by ATRA in this cell line (Figure 1A).

Since the phosphorylation of Y174 is known to be involved in functions played by Vav1 in mature neutrophils, a possible role for this residue also in the completion of the differentiation program of NB4 cells was investigated. With this aim, a construct containing the Y174F mutated cDNA for Vav1 was transiently expressed in NB4 cells prior to their treatment with ATRA. The evaluation of differentiation-related features showed a slight but not significant increase of both CD11b expression (Figure 1B) and migration capability (Figure 1C) in cells overexpressing the Y174F mutated Vav1.

To establish if the ATRA-induced phosphorylation of Vav1 on Y174 may depend on the tyrosine kinase Syk, we have performed immunochemical analysis on Vav1 immunoprecipitated from NB4 cells induced to differentiate in the presence

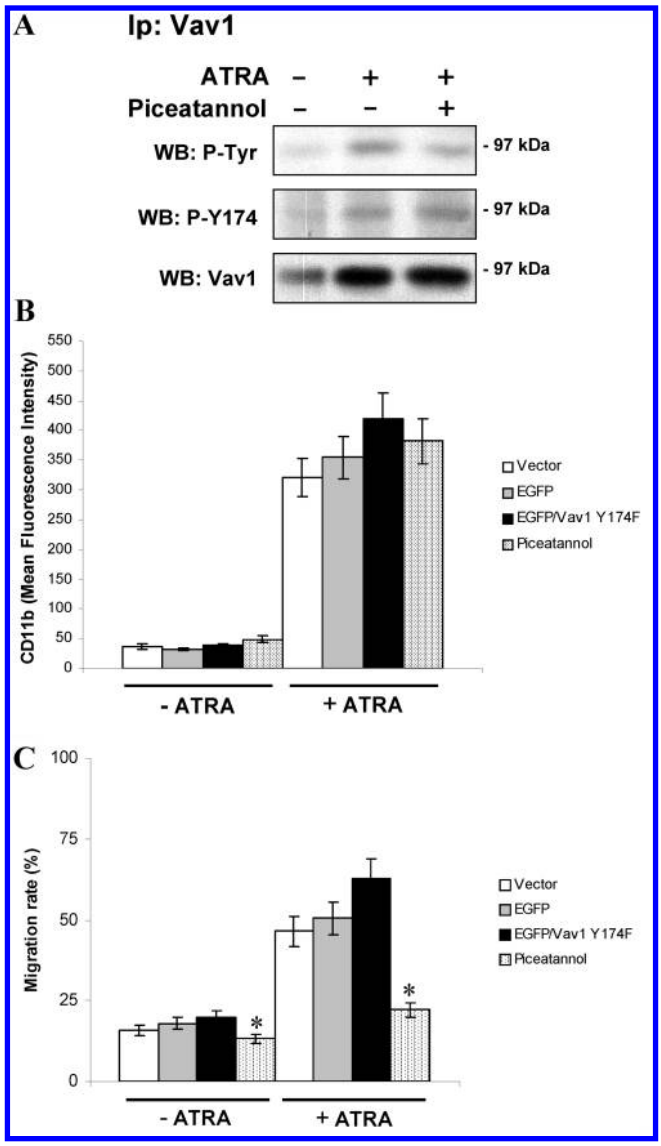

Figure 1. Effects of mutation of Vav1 Y174 on differentiation of NB4 cells. (A) Immunochemical analysis with the indicated antibodies of Vav1 immunoprecipitated from NB4 cells cultured in the absence (-) or in the presence (+) of ATRA and/or of the Syk inhibitor Piceatannol. The data are representative of 2 separate experiments performed in duplicate. NB4 cells transiently co-transfected with plasmids containing a cDNA for EGFP and a cDNA for Vav1 mutated in the sequence encoding for Y174 (Vav1 Y174F) or treated with Piceatannol were cultured in control (-ATRA) and differentiating (+ATRA) conditions then subjected to analysis of CD11b expression (B) and migration assays (C). The migration rate was expressed as percentage of migrated cells on the total cell number seeded in the upper compartment of the chamber. The asterisks indicate statistically significant values. The data are representative of 3 separate experiments ± SD.

of Piceatannol, a specific inhibitor of Syk activity. As reported in Figure 1A, although the total tyrosine phosphorylation level of Vav1, as expected, was reduced when Syk activity was inhibited, no significant effects of Piceatannol on the phosphorylation of Y174 were observed. The analysis of differentiation-related features indicated that, as expected, Piceatannol was ineffective on CD11b expression (Figure 1B) but its administration in conjunction with ATRA considerably reduced the agonist-induced migration capability of NB4 cells (Figure 1C).

Identification of the Y745 of Vav1 as a Phosphorylated Tyrosine in Differentiating NB4 Cells. Since the Y174 residue of Vav1 does not seem crucial for the ATRA-induced maturation of NB4 cells, our study was then aimed to identify tyrosine phosphorylated residue(s) that play(s) a key role in the completion of differentiation to neutrophils of APL-derived precursors.

Even if ATRA treatment induces, in our cell model, a significant increase in the amount of Vav1, it remains a relatively low-abundance protein in the cell. To improve at least in part the recovery of Vav1, we employed the MicroRotofor apparatus for IEF in free solution. After separation of NB4 crude extracts by MicroRotofor, proteins were subjected to monodimensional electrophoresis followed by immunochemical analysis with the anti-Vav1 antibody. As reported in Figure 2A, in both control and differentiated conditions, Vav1 was detected in fractions matching the 7.9−10 pH range and, as expected, its amount increased after ATRA treatment.

The protein was then immunoprecipitated from the pull of the Vav1-containing fractions (fractions 8−10) and, after monodimensional electrophoresis and staining of the polyacrylamide gel, the bands corresponding to Vav1 were excised and subjected to mass spectrometry analysis. The presence of Vav1 in the immunoprecipitates was confirmed by immunochemical analysis with the specific antibody performed on equivalent protein samples transferred to a nitrocellulose membrane (Figure 2B).

Mass spectrometry analysis of peptide mixtures originated by trypsin digestion of Vav1 immunoprecipitated from control and ATRA treated NB4 cells allowed to identify, in both conditions, human Vav1 by matching 13 experimental peptides (Table 1). Moreover, in both samples from control and differentiated cells, two different peaks, shifted by 80 Da, corresponded to the same peptide covering the 737−755 aminoacidic sequence of Vav1 (Figure 3A), indicating the presence of a phosphopeptide (HPO_3 = 80 Da). As deduced by multiple sequence alignment, the identified peptide is a conserved aminoacidic sequence and contains a highly conserved tyrosine (Figure 3B), corresponding to the residue 745 of human Vav1.

A further set of experiments was then performed to establish if Y745 of Vav1 is actually phosphorylated during differentiation of NB4 cells. A plasmid expressing a mutated form of Vav1, in which tyrosine 745 was replaced with phenylalanine (Y745F), was originated by *in situ* mutagenesis and used to transiently transfect NB4 cells, in parallel with a construct overexpressing wild-type Vav1 (Vav1 WT). Since the latter included a Myc-tail, immunochemical analysis performed with the anti-Vav1 antibody allowed to distinguish the native from the overexpressed protein (Figure 4A).

Transfected cells were treated with ATRA and the overexpressed wild-type and Y745F mutated Vav1 were immunoprecipitated with an anti-Myc antibody (Figure 4B). After monodimensional separation and gel staining, the extracted proteins were subjected to mass spectra analysis. Also in this case, human Vav1 was identified in immunoprecipitated wild-type and mutated proteins through peptide mass fingerprinting (Table 2).

The analysis of mass spectra acquired by MALDI-TOF/TOF indicated that the peptide m/z = 2399.024 shown by the overexpressed wild-type Vav1 (Figure 4C, Vav1 WT) corresponds to the putative phosphorylated peptide found in the

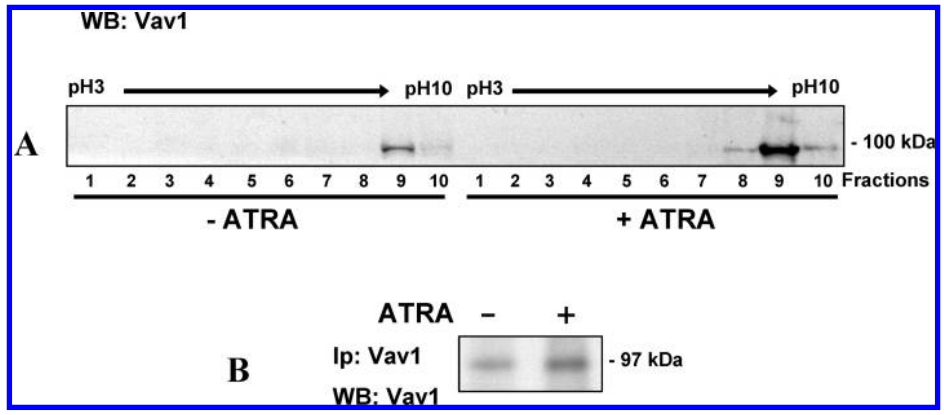

Figure 2. Purification of Vav1 from NB4 differentiating cells. (A) Immunochemical analysis with an anti-Vav1 antibody of protein fractions obtained after separation with MicroRotofor of lysates from control (-ATRA) and differentiated (+ATRA) NB4 cells. (B) Immunochemical analysis of Vav1 immunoprecipitated from protein fractions 8, 9, and 10 obtained with MicroRotofor from control (-) and differentiated (+) NB4 cells. The data are representative of 2 separate experiments performed in duplicate.

Table 1. Identification of Vav1 by Peptide Mass Fingerprint after Immunoprecipitation of the Protein from Differentiating NB4 Cells

sample	NCBI accession number	identified protein	Mascot score	sequence coverage	matching peptides	theoretical M_r/pI
Control	gi|119589464	Vav 1	90	25%	13/25	99.2 kDa/6.7
ATRA	gi|119589464	Vav 1	84	25%	13/30	99.2 kDa/6.7

native protein reported in Figure 3A, having a theoretical m/z = 2399.09, with a mass error of 0.066 ppm. As reported in Figure 4C, the signal is evident in the spectrum originated by over-expressed wild-type Vav1 (Vav1 WT) and almost absent in the spectrum from overexpressed mutated Vav1 (Vav1 Y745F), although in this case more shots were added (9000 instead of 7600).

The analysis of the fragments originated by the peptide having m/z = 2399.024 from overexpressed wild-type Vav1 (Vav1 WT) allowed to establish that the tyrosine 745 of Vav1 is actually phosphorylated, as deduced by the peak at m/z = 216.018, corresponding to the tyrosine immonium ion (Figure 4D).

Tyrosine 745 of Vav1 Is Involved in ATRA-Induced Differentiation of NB4 Cells. The subsequent part of the work was designed to establish if the residue Y745 of Vav1 plays a role in the differentiation process of NB4 cells. With this aim, maturation-related features, like the expression of the surface antigen CD11b and the acquisition of migration capability, were evaluated in NB4 cells induced to differentiate with ATRA when Y745 of Vav1 was replaced with phenylalanine. NB4 cells were therefore transiently co-transfected with a plasmid containing the full-length cDNA of human wild-type (Vav1 WT) or mutated (Vav1 Y745F) Vav1 and with a plasmid containing the cDNA for EGFP and then induced to differentiate with ATRA (Figure 5A). The immunochemical analysis of transfected cells performed with the anti-Vav1 antibody confirmed the presence of both native and overexpressed proteins (Figure 5A). The use of the anti-phosphotyrosine antibody failed to reveal any phosphorylation of the overexpressed Y745F mutated Vav1 (Figure 5A).

The cytofluorimetrical analysis performed exclusively on the EGFP-labeled green cells (overexpressing cells) showed that the overexpression of Vav1 WT induced a slight increase in CD11b expression, confirming our previous results. On the contrary, when the Y745F mutated form of Vav1 was overexpressed, the ability of ATRA of inducing CD11b expression was strongly reduced (Figure 5B).

Since granulocytic differentiation is associated with the acquisition of migratory capabilities, transfected NB4 cells were subjected to migration assays. As reported in Figure 5C, ATRA treatment induced, as expected, an increase of the migration rate of both control and EGFP-transfected cells. The analysis restricted to the green cells (overexpressing cells) showed that the overexpression of Vav1 WT induced a slight but significant increase of the migration capability of both control and ATRA-treated NB4 cells (Figure 5C). Notably, the mutation of the Vav1 Y745 strongly reduced the ability of differentiating tumor promyelocytes to cross the porous membrane in response to ATRA-treatment (Figure 5C).

Discussion

Vav1 is a hematopoietic cell specific isoform of the Vav family of Rho/Rac GEFs, activated by tyrosine phosphorylation,[15] that is involved in both maturation and functions of lymphocytes[16] and in agonist induced response of mature myeloid cells, including neutrophils.[19]

Our previous data support the notion that Vav1 plays crucial functions in the maturation process of tumoral promyelocytes, since we have demonstrated that the down-modulation of Vav1 prevents, while its overexpression promotes, the ATRA-induced differentiation of APL-derived cells.[11] In particular, we found that Vav1, up-regulated and tyrosine phosphorylated during maturation of both APL blasts and APL-derived cell lines, plays a role in modulating ATRA-dependent gene and protein expression,[11,13] possibly as part of molecular complexes with transcription factors known to regulate the ATRA-induced differentiation process to neutrophils.[14]

Stemming from these evidence, this work aimed to identify tyrosine(s) residue(s) whose phosphorylation is functionally involved in the role(s) played by Vav1 in response to ATRA treatment of APL-derived promyelocytes.

In both lymphoid and myeloid cells, including neutrophils, it has been reported that the highly conserved Y174 residue of Vav1 can bind to, and become phosphorylated by, tyrosine kinases such as Lck and Syk/ZAP-70.[13,16] This event is mainly associated to GEF activity, but recent data obtained in both lymphoid and nonlymphoid cells suggest that Y174 is also involved in roles of Vav1 not mediated by GEF activity.[9,27] Here

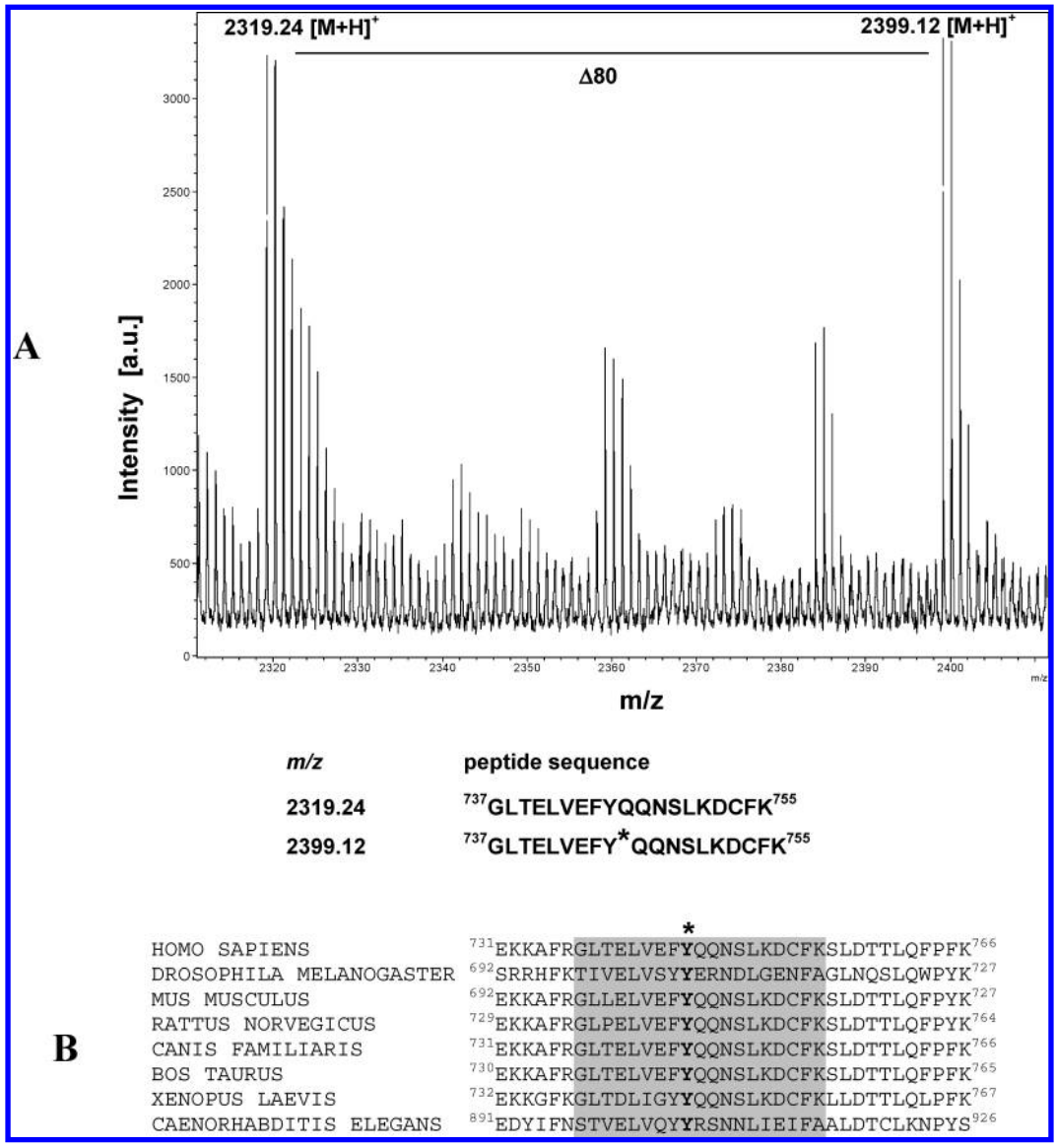

Figure 3. Identification of a putative Vav1 phosphorylated tyrosine by mass spectrometry. (A) Magnified view of the mass spectrum obtained from differentiated cells, showing two peaks at *m*/*z* corresponding to the isotopic pattern of both modified (*m*/*z* = 2399.12) and unmodified (*m*/*z* = 2319.24) peptide, whose sequences are reported below. A Δ*m*/*z* of 80 suggests the presence of a phosphorylated residue. The asterisk identifies the putative phosphorylated tyrosine (Y745). The data are representative of 3 separate experiments. (B) Protein multiple sequence alignment, produced with ClustalW 1.81, of Vav1 protein from *Homo sapiens*, *Drosophila melanogaster*, *Mus musculus*, *Rattus norvegicus*, *Canis familiaris*, *Bos taurus*, *Xenopus laevis* and *Caenorhabditis elegans*. The conserved amino acid sequences, including the 737−755 peptide of human Vav1 identified by mass spectrometry, are shaded in gray and the highly conserved tyrosine is in bold and marked with an asterisk at the top of the alignment.

we have found that Y174 of Vav1 increases its phosphorylation level in NB4 cells after ATRA treatment. On the other hand, both cytofluorimetrical analysis of CD11b expression and migration assays ruled out a crucial role for this tyrosine residue in regulating the agonist-induced maturation process of this cell line.

We have previously demonstrated that, in APL-derived cells, tyrosine phosphorylation of Vav1 is at least in part dependent on the tyrosine kinase Syk, known to be activated in mature neutrophils, in which it regulates the formation of lamellipodia during phagocytosis.[22] By administering ATRA in the presence of Piceatannol, a specific inhibitor of Syk activity, here we have found that Y174 is not phosphorylated by this kinase during differentiation of NB4 cells. In fact, although both the Y174F mutation and Piceatannol administration do not have any effect on CD11b expression, only the Syk inhibitor impairs the migratory capability of ATRA-treated NB4 cells.

Since phosphorylation of Y174 in neutrophils was mainly associated to the GEF activity of Vav1 in mature cells, these findings suggest that the ATRA-induced phosphorylation of Y174 occurs in parallel with the acquisition of a mature phenotype instead of being directly involved in the differentiation process of NB4 cells. These data are also in agreement with our previous results indicating that Vav1 is not involved in the increase of total GEF activity on small G proteins induced by ATRA in HL-60 promyelocytes.[24]

Vav1 contains a total of 31 tyrosine residues, and in addition to the highly conserved Y174, other residues may be involved in roles played by Vav1 in our cell model. To address this issue, a more accurate analysis of Vav1 tyrosine(s) residue(s) phos-

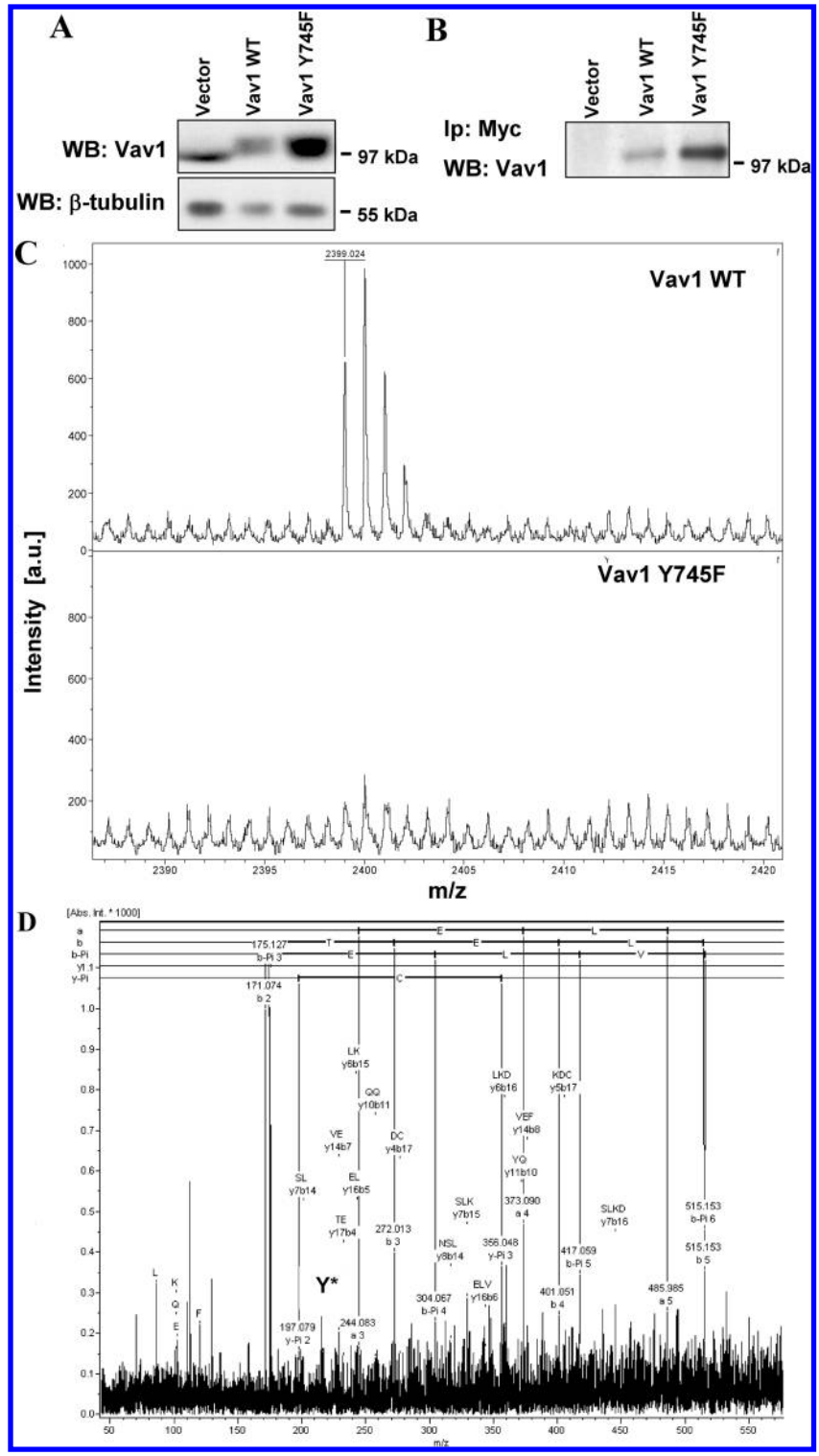

Figure 4. Effects of ATRA on phosphorylation of the Vav1 Y745 residue. (A) NB4 cells, transiently transfected with a plasmid containing a Myc-tagged wild-type cDNA for Vav1 (Vav1 WT) or the Vav1 cDNA mutated in the sequence encoding for Y745 (Vav1 Y745F) were cultured in differentiating conditions and subjected to immunochemical analysis with the indicated antibodies. The analysis of β-tubulin content allowed to compare the amounts of loaded proteins. (B) Overexpressed wild-type (Vav1 WT) or mutated (Vav1 Y745F) Vav1 was immunoprecipitated from ATRA-treated NB4 cells by using an anti-Myc antibody able to recognize the Myc tail of overexpressed proteins. (C) Magnified view of mass spectra acquired by MALDI-TOF/TOF from wild-type (Vav1 WT) or mutated (Vav1 Y745F) Vav1 immunoprecipitated from ATRA-treated NB4 cells. The peptide $m/z = 2399.024$ corresponds to the putative phosphorylated peptide. (D) Part of the spectrum acquired in MS/MS. The signal at $m/z = 216.018$ corresponds to the ion immonium of Y* (Y745). All the data are representative of 3 separate experiments.

phorylated during the completion of the maturation program of APL-derived cells was performed. By mass spectra analysis of Vav1 immunoprecipitated from control and ATRA treated NB4 cells, we have identified, in both conditions, Y745 as a phosphorylated residue within a highly conserved Vav1 sequence. MALDI-TOF/TOF analysis on Y745F mutated Vav1

Table 2. Protein Identification by Peptide Mass Fingerprint after Overexpression of Wild-Type (Vav1 WT) or Mutated (Vav1 Y745F) Vav1 in Differentiating NB4 Cells

sample	NCBI accession number	identified protein	Mascot score	sequence coverage	matching peptides	theoretical M_r/pI
Vav1 WT	gi|119589464	Vav 1	84	17%	11/23	99.2 kDa/6.7
Vav1 Y745F	gi|119589464	Vav 1	68	14%	9/26	99.2 kDa/6.7

overexpressed in differentiating cells indicated that phosphorylation of this tyrosine residue is induced by ATRA. It can be thus speculated that ATRA induces the increase of the phosphorylation level of tyrosine residues already phosphorylated at basal levels in untreated NB4 cells, according to the notion that retinoids act in APL-derived cells mainly by promoting the completion of an already initiated maturation program.

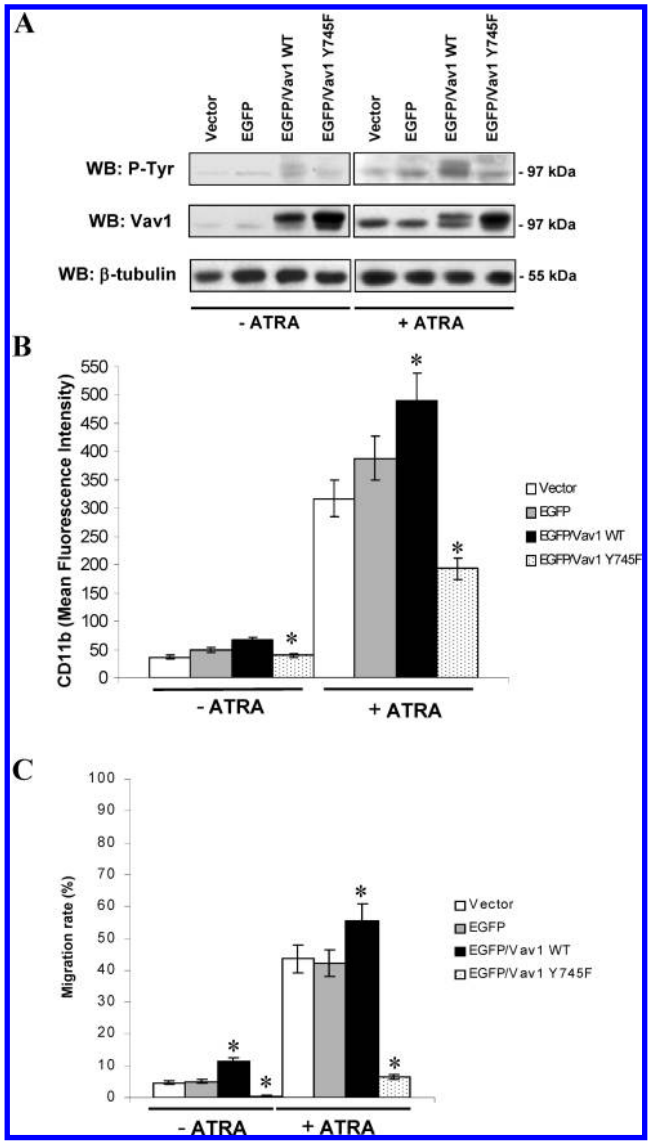

Figure 5. Effects of mutation of Vav1 Y745 on differentiation of NB4 cells. NB4 cells were transiently co-transfected with a plasmid codifying for EGFP and with a construct containing the cDNA for the wild-type (Vav1 WT) or for the Y745F mutated (Vav1 Y745F) Vav1 and then cultured in control (-ATRA) or differentiating (+ATRA) conditions. Transfected cells were subjected to immunochemical analysis with the indicated antibodies (A), to cytofluorimetrical analysis of CD11b expression (B) and to migration assays (C). The analysis of β-tubulin content allowed to check for the amount of loaded protein. Migration rate was expressed as percentage of migrated cells on the total cell number seeded in the upper compartment of the chamber. The asterisks indicate statistically significant values. The data are representative of 3 separate experiments ± SD.

To elucidate whether the ATRA induced increase in the phosphorylation level of Y745 is directly involved in the maturation process, differentiation-related features were analyzed in differentiating NB4 cells overexpressing the Y745F mutated form of Vav1. The obtained results showed that tyrosine phosphorylation of Vav1 Y745 plays a crucial role in regulating CD11b expression as well as in promoting the acquisition of migratory capability.

By administering ATRA in the presence of Piceatannol, we ruled out the possibility that Y745 is phosphorylated by Syk during differentiation of NB4 cells. In fact, in contrast with the inhibition of Syk activity, Y745F mutation negatively affects CD11b expression of differentiating cells.

Considering that GEF activity of Vav1 in mature neutrophils is chiefly regulated by Syk, it can be speculated that the phosphorylation of Y745 might be an important step of the mechanism of activation of this protein in promyelocytes that are completing their maturation program, ended to mediate functions of Vav1 other than guanosine exchange factor.

In addition to Y174, other conserved residues, Y142 and Y160, have been described to be phosphorylated in activated Vav1. It was also suggested that phosphorylation of the tyrosines located inside the acidic region of Vav1 may allow Y142, Y160, and Y174 to become docking sites for kinases, which can then phosphorylate additional tyrosine residues in Vav proteins.[27]

Multiple sequence alignment analysis of proteins from different species indicates that Y745 of the human Vav1 is a highly conserved amino acid, suggesting its functional involvement in physiological roles of Vav1. However, this Vav1 residue has never been described before and, to our knowledge, never has been associated to known pathologies.

By means of softwares designed to predict cell signaling interactions using short sequence motifs, a number of tyrosines, in addition to the highly conserved AC residues, have been found to be located within known consensus motifs for phosphorylation.[28] The same analysis failed to recognize the Y745 residue as a putative phosphorylation site by the known tyrosine kinases. However, some tyrosine residues of Vav1 are not surface exposed and/or may be involved in intramolecular interactions, thereby precluding their tyrosine phosphorylation and impairing their recognition by the database analysis. Since Y745 is located inside a short helix on the SH2 domain of Vav1, its phosphorylation could be an event secondary to phosphorylation of other tyrosine residues, which may induce conformational changes of Vav1 allowing Y745 to become accessible to a specific tyrosine kinase.

Our work has identified a not yet described Vav1 tyrosine, Y745, as a crucial residue in regulating the acquisition of a differentiated phenotype by ATRA-treated tumoral myeloid precursors. We expect that our findings will contribute to better define the involvement of Vav1 in the maturation process of

APL promyelocytes and to identify new specific targets for therapy of this myeloid leukemia.

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References

(1) Mistry, A. R.; Pedersen, E. W.; Solomon, E.; Grimwade, D. The molecular pathogenesis of acute promyelocytic leukaemia: implications for the clinical management of the disease. *Blood* **2003**, *17*, 71–97.
(2) Wojiski, S.; Guibal, F. C.; Kindler, T.; Lee, B. H.; Jesneck, J. L.; Fabian, A.; Tenen, D. G.; Gilliland, D. G. PML-RARalpha initiates leukemia by conferring properties of self-renewal to committed promyelocytic progenitors. *Leukemia* **2009**, *23*, 1462–71.
(3) Lo-Coco, F.; Ammatuna, E. The biology of acute promyelocytic leukemia and its impact on diagnosis and treatment. *Hematol. Am. Soc. Hematol. Educ. Program* **2006**, *2006*, 156–61.
(4) Tallman, M. Treatment of relapsed or refractory acute promyelocytic leukemia. *Best Pract. Res., Clin. Haematol.* **2007**, *20*, 57–65.
(5) Wang, Z. Y.; Chen, Z. Acute promyelocytic leukemia: from highly fatal to highly curable. *Blood* **2008**, *111*, 2505–15.
(6) Breitman, T. R.; Selonick, S. E.; Collins, S. J. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2936–40.
(7) Lanotte, M.; Martin-Thouvenin, V.; Najman, S.; Balerini, P.; Valensi, F.; Berger, R. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). *Blood* **1991**, *77*, 1080–6.
(8) Lee, K. H.; Chang, M. Y.; Ahn, J. I.; Yu, D. H.; Jung, S. S.; Choi, J. H.; Noh, Y. H.; Lee, Y. S.; Ahn, M. J. Differential gene expression in retinoic acid-induced differentiation of acute promyelocytic leukemia cells, NB4 and HL-60 cells. *Biochem. Biophys. Res. Commun.* **2002**, *296*, 1125–33.
(9) Katzav, S. Vav1: a hematopoietic signal transduction molecule involved in human malignancies. *Int. J. Biochem. Cell Biol.* **2009**, *41*, 1245–8.
(10) Bertagnolo, V.; Brugnoli, F.; Marchisio, M.; Capitani, S. Inositide-modifying enzymes: a cooperative role in regulating nuclear morphology during differentiation of myeloid cells. *J. Biol. Regul. Homeostatic Agents* **2004**, *18*, 381–6.
(11) Bertagnolo, V.; Brugnoli, F.; Mischiati, C.; Sereni, A.; Bavelloni, A.; Carini, C.; Capitani, S. Vav promotes differentiation of human tumoral myeloid precursors. *Exp. Cell. Res.* **2005**, *306*, 56–63.
(12) Zhang, R.; Tsai, F. Y.; Orkin, S. H. Hematopoietic development of vav−/−mouse embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 12755–9.
(13) Bertagnolo, V.; Grassilli, S.; Bavelloni, A.; Brugnoli, F.; Piazzi, M.; Candiano, G.; Petretto, A.; Benedusi, M.; Capitani, S. Vav1 modulates protein expression during ATRA induced maturation of APL derived promyelocytes: a proteomic-based analysis. *J. Proteome Res.* **2008**, *7*, 3729–36.
(14) Brugnoli, F; Lambertini, E; Varin-Blank, N; Piva, R; Marchisio, M; Grassilli, S; Miscia, S; Capitani, S; Bertagnolo, V. Vav1 and PU.1 are recruited to the *CD11b* promoter in APL-derived promyelocytes: role of Vav1 in modulating PU.1 containing complexes during ATRA induced differentiation. *Exp. Cell Res.* **2010**, *316*, 38–47.
(15) Bustelo, X. R. Regulation of Vav proteins by intramolecular events. *Front. Biosci.* **2002**, *7*, d24–d30.
(16) Tybulewicz, V. L. Vav-family proteins in T-cell signaling. *Curr. Opin. Immunol.* **2005**, *17*, 267–74.
(17) Katzav, S. Flesh and blood: the story of Vav1, a gene that signals in hematopoietic cells but can be transforming in human malignancies. *Cancer Lett.* **2007**, *255*, 241–54.
(18) Kuhne, M. R.; Ku, G.; Weiss, A. A guanine nucleotide exchange factor-independent. *J. Biol. Chem.* **2000**, *275*, 2185–90.
(19) Zhao, T.; Benard, V.; Bohl, B. P.; Bokoch, G. M. The molecular basis for adhesion-mediated suppression of reactive oxygen species generation by human neutrophils. *J. Clin. Invest.* **2003**, *104*, 1732–40.
(20) Gakidis, M. A.; Cullere, X.; Olson, T.; Wilsbacher, J. L.; Zhang, B.; Moores, S. L.; Ley, K.; Swat, W.; Mayadas, T.; Brugge, J. S. Vav GEFs are required for beta2 integrin-dependent functions of neutrophils. *J. Cell. Biol.* **2004**, *166*, 273–82.
(21) Fumagalli, L.; Zhang, H.; Baruzzi, A.; Lowell, C. A.; Berton, G. The Src family kinases Hck and Fgr regulate neutrophil responses to N-formyl-methionyl-leucyl-phenylalanine. *J. Immunol.* **2007**, *178*, 3874–85.
(22) Schymeinsky, J.; Sindrilaru, A.; Frommhold, D.; Sperandio, M.; Gerstl, R.; Then, C.; Mócsai, A.; Scharffetter-Kochanek, K.; Walzog, B. The Vav binding site of the non-receptor tyrosine kinase Syk at Tyr 348 is critical for beta2 integrin (CD11/CD18)-mediated neutrophil migration. *Blood* **2006**, *108*, 3919–27.
(23) Bertagnolo, V.; Marchisio, M.; Brugnoli, F.; Bavelloni, A.; Boccafogli, L.; Colamussi, M. L.; Capitani, S. Requirement of tyrosine-phosphorylated Vav for morphological differentiation of all trans-retinoic acid-treated HL-60 cells. *Cell Growth Differ.* **2001**, *12*, 193–200.
(24) Bertagnolo, V.; Brugnoli, F.; Marchisio, M.; Celeghini, C.; Carini, C.; Capitani, S. Association of PI3-K with tyrosine phosphorylated Vav is essential for its activity in neutrophil-like maturation of myeloid cells. *Cell. Signalling* **2004**, *16*, 423–33.
(25) Bertagnolo, V.; Marchisio, M.; Volinia, S.; Caramelli, E.; Capitani, S. Nuclear association of tyrosine-phosphorylated Vav to phospholipase C-gamma1 and phosphoinositide 3-kinase during granulocytic differentiation of HL-60 cells. *FEBS Lett.* **1998**, *441*, 480–4.
(26) Brugnoli, F.; Bavelloni, A.; Benedusi, M.; Capitani, S.; Bertagnolo, V. PLC-beta2 activity on actin-associated polyphosphoinositides promotes migration of differentiating tumoral myeloid precursors. *Cell. Signalling* **2007**, *19*, 1701–12.
(27) Miletic, A. V.; Sakata-Sogawa, K.; Hiroshima, M.; Hamann, M. J.; Gomez, T. S.; Ota, N.; Kloeppel, T.; Kanagawa, O.; Tokunaga, M.; Billadeau, D. D.; Swat, W. Vav1 acidic region tyrosine 174 is required for the formation of T cell receptor-induced microclusters and is essential in T cell development and activation. *J. Biol. Chem.* **2006**, *281*, 38257–65.
(28) Obenauer, J. C.; Cantley, L. C.; Yaffe, M. B. Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic Acids Res.* **2003**, *31*, 3635–41.

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