Peer-reviewed version available at J. Clin. Med. 2019, 8, 972; doi:10.3390/jcm8070972

Type of paper: Article

Reduced expression of Sprouty1 contributes to the aberrant proliferation and impaired apoptosis of acute myeloid leukemia cells

Valentina Rosso[1,#], Cristina Panuzzo[1,#], Jessica Petiti[1], Sonia Carturan[1], Matteo Dragani[1], Giacomo Andreani[1], Carmen Fava,[1] Giuseppe Saglio[1], Enrico Bracco[2,$] and Daniela Cilloni[1,$,*]

[1]Department of Clinical and Biological Sciences, University of Turin, Turin, Italy. valentina.rosso@gmail.com, cristina.panuzzo@unito.it, jessica.petiti@unito.it, sonia.carturan@unito.it, matteo.dragani@gmail.com, giacomo.andreani@unito.it, carmen.fava@unito.it, giuseppe.saglio@unito.it, daniela.cilloni@unito.it

[2]Department of Oncology, University of Turin, Turin, Italy. enrico.bracco@unito.it

[#] VR and CP contributed equally to this manuscript

[$] EB and DC contributed equally to this manuscript

* Corresponding author: Daniela Cilloni, M.D - Dept of Clinical and Biological Sciences of the University of Turin - San Luigi Hospital, Regione Gonzole 10, 10043 ORBASSANO-TORINO, ITALY. Tel +39-011-9026610; Fax +39-11-9038636; e-mail: daniela.cilloni@unito.it

Abstract: In most of acute myeloid leukemia patients there is an aberrant tyrosine kinases activity. The Sprouty family proteins were originally identified in *Drosophila melanogaster* as antagonists of Breathless, the mammalian ortholog of fibroblast growth factor receptor. This family proteins are inhibitors of RAS signaling induced by tyrosine kinases receptors and they are implicated in negative feedback processes regulating several intracellular pathways.

The present study aims to investigate the role of a member of the Sprouty family, Sprouty1, as regulator of cell proliferation and growth in patients affected by acute myeloid leukemia. Sprouty1 mRNA and protein were both significantly down-regulated in acute myeloid leukemia cells compared to the normal counterpart, but they were restored when remission is achieved after chemotherapy. Ectopic expression of Sprouty1 revealed that it plays a key role in the proliferation and apoptotic defect that represent a landmark of the leukemic cells. Our study identified Sprouty1 as negative regulator involved in the aberrant signals of acute myeloid leukemia. Furthermore, we found a correlation between Sprouty1 and FoxO3a delocalization in AML at diagnosis, suggesting a multistep regulation of RAF–MEK–ERK signaling in human cancers.

Keywords: Sprouty1, AML, FoxO3a

Peer-reviewed version available at J. Clin. Med. 2019, 8, 972; doi:10.3390/jcm8070972

1. Introduction

Acute myeloid leukemia (AML) develops from malignant transformation of immature hematopoietic cells through a complex multistep process that requires cooperation of different genetic alterations [1]. In most of the AML patients there is an aberrant tyrosine kinase (TK) activity, which results into an impaired differentiation, altered cell growth and apoptosis defect. Sprouty proteins inhibit the RAF–MEK-ERK pathway, frequently constitutively active in many human tumors, contributing robustly to cells aggressiveness and invasion [2-5].

The Sprouty family proteins were initially identified in *Drosophila melanogaster* as antagonists of receptor tyrosine kinase (RTK) signaling during different morphogenetic processes, including the development of the trachea, the eye, the wing and other tissues [6-11].

The biological functions of the Sprouty proteins have been attributed to its conserved motifs: all Sprouty proteins share a characteristic Cys-rich C-terminus domain (SPRY domain), which is believed to be indispensable for their function [12,13].

Sprouty proteins have been implicated in the regulation of the biological processes responsible of tumor growth, development and metastasis, including cell proliferation, migration, invasion and survival. Down regulation of Sprouty1 has been found in carcinomas of the breast, prostate cancer, leukemia [14] and renal cell carcinoma [3].

Experimental evidences showed that Sprouty can paradoxically act either as negative or positive regulator of the tumor progression [15-18]. The presence of mutations on RAS cascade has also been shown to be an important determinant of the Sprouty's deregulated action [19]. Different studies described an interaction between FoxO3a and Sprouty family proteins [20-22].

FoxO3a belong to the family of forkhead transcription factors, which are characterized by the presence of a DNA binding region highly conserved called "forkhead box" [23]. Human forkhead proteins are represented by 4 members: FoxO1, FoxO3a, FoxO4 and FoxO6 and are normally present in an active form in the nucleus. The FoxO proteins have partially overlapping functions: their target genes are involved in processes such as cell cycle arrest [24-26], DNA repair [25,27], cell differentiation [28], apoptosis [29-31] and homeostasis of the hematopoietic system, through the regulation of HSC compartment [32]. FoxO family operates under the negative control of Akt: in response to the binding of growth factors (e.g. insulin) to their membrane receptors, the PI3K is activated. The activated Akt in turn then phosphorylates FoxO proteins, resulting in the inactivation of these transcription factors and in their translocation from the nucleus to the cytosol. Moreover, in breast cancer the cytoplasmic localization of FoxO3a is correlated with poor survival [33]. Similarly, in leukemia patients FoxO3a phospho-status correlates with some clinical features and overall survival [34], suggesting a pivotal role of FoxO proteins in cancer cells.

In this study we investigated the role of Sprouty1 as regulator of cell proliferation and growth in patients affected by acute myeloid leukemia and we studied the correlation between low Sprouty1 expression and FoxO3a delocalization in AML at diagnosis, suggesting a multistep regulation of RAF–MEK–ERK signaling in human cancers.

Peer-reviewed version available at J. Clin. Med. 2019, 8, 972; doi:10.3390/jcm8070972

2. Materials and Methods

2.1. Patients and cell lines

After written informed consent (number of approval 201/2014), 82 bone marrow (BM) and 8 peripheral blood (PB) specimens from AML patients at diagnosis, 15 PB from AML patients after therapy and 16 BM and 18 PB from healthy subjects were collected. All the patients have been previously characterized at the cytogenetic level by conventional karyotyping and screened by reverse transcriptase-PCR for the presence of the most frequent fusion transcripts. Mutations or internal tandem duplication of both FLT3 and of NPM1 genes were also characterized. Acute promyelocytic leukemia samples were excluded from the study.

The human Kasumi-1 cell line was purchased from ATCC and cultured in RPMI-1640 supplemented with 20% fetal bovine serum (FBS), 500 U/ml penicillin and 0.5 mg/ml streptomycin. Cells were cultured at 37°C in a humidified atmosphere flushed with 5% CO_2.

2.2. RNA Extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol Reagent (Ambion, Thermo Fisher Scientific), according to the manufacturer's instructions. 1 µg of total RNA was reverse transcribed using random hexamers as primers in a final volume of 25 µL. For Sprouty1 mRNA quantification, specific assays (assay ID for ABL Hs00245445_m1, and Hs00544790_m1 for Sprouty1 - Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA) were used according to the manufacturer's instructions. The analysis was performed in triplicate. The Sprouty1 Cts obtained by qRT-PCR were normalized with respect to the Ct of ABL and expressed as $2^{-\Delta\Delta Ct}$. Universal human references RNA (Stratagene, San Diego) was used to calibrate the assay.

2.3. Cells lysis

For total cell extracts, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with RIPA buffer on ice (10% glycerol; 1% Triton X-100; 20mM Hepes pH 7.4; 5mM EDTA pH7.2; 150mM NaCl) supplied with protease and phosphatase inhibitors (1mM Na3VO4, 1mM PMSF, 2µg/ml leupeptin, 2µg/ml aprotinin, 2µg/ml pepstatin). For nuclear and cytoplasmatic extracts, cells were washed with ice-cold PBS and incubated on ice in 600µl of cytosolic lysis buffer (10mM Hepes Ph7.9; 10mM KCl; 0.1mM EDTA; 0.5% NP40; 1µg/ml leupeptin, 1µg/ml aprotinin, 1µg/ml pepstatin; 1mM Na3VO4, 100 µg/ml PMSF). After 30 min, nuclei were separated by centrifugation at 3000xg for 10 min and the supernatants collected (cytoplasmic fraction). Nuclei pellets were resuspended in 100µl of nuclear lysis buffer (20mM Hepes pH 7.9; 400mM KCl; 1mM EDTA; 1mM EGTA; 1mM DTT; 10% glycerol; 1µg/ml leupeptin, 1µg/ml aprotinin, 1µg/ml pepstatin; 100 µg/ml PMSF) and incubated on ice for 20 min with vigorous mixing. The nuclear lysates were further clarified by high-speed centrifugation.

2.4. Western blot analysis

Seventy µg of total proteins were loaded and run onto 10% SDS-PAGE and transferred to PVDF (Bio-Rad) membranes. Membranes were blocked in TBS (Tris-HCl pH7.4, 150mM NaCl) plus 5% BSA for 1 hr at room temperature (RT) and then decorated with appropriate antibodies (Sprouty1 sc-365520 and Tubulin sc-23948, Sanza Cruz Biotecnology; TATA Binding protein (TBP) MA1-189

Peer-reviewed version available at J. Clin. Med. 2019, 8, 972; doi:10.3390/jcm8070972

and Vinculin MA5-11690, Sigma-Aldrich; Foxo3a #2497, Cell Signaling) in PBS-Tween 0.2% over night at 4°C. Membranes were than washed with PBS-Tween 0.2% three times for 15 min each, incubated with appropriate peroxidase-linked secondary antibody (Sanza Cruz Biotecnology) for 1 hr at RT and washed again in PBS-Tween 0.2%. Specific binding was detected using an enhanced chemiluminescence system (Clarity Western ECL Substrate #170-5061, Bio-Rad).

2.5. Immunofluorescence assay

Cytospins were prepared using BM cells from AML patients at diagnosis or in remission phase and Kasumi-1 cell line. Cells were fixed with 4% PFA, permeabilized and blocked for 45 min. Then, cells were incubated for 2 hr at RT with polyclonal anti-Sprouty1 or polyclonal anti-FoxO3a antibodies. Detection of proteins was obtained by incubation for 30 min with a secondary antibodies. Cells were then incubated for 5 min with propidium iodide for nuclear staining and analyzed with confocal scanning microscope (LSM 5110; Carl Zeiss MicroImaging Inc.). Images were captured using 63X objective. Fluorescent signal was measured by image processing (LSM800) and analyzed in Java (Image J) program https://imagej.nih.gov/ij/download.html.

2.6. Plasmid construction and trasfection

pCGN-Sprouty1 and pECE-FoxO3a (kindly donated by Prof. PP Pandolfi) vectors were used for transient transfection of Kasumi-1 cells by FuGENE-6 (Roche Applied Science), according to the manufacturer's instructions.

The simultaneous transfection with pEGFP-C2 vector alone allowed to check the trasfection efficiency after 48 hr.

2.7. Proliferation and apoptosis assays

Cell growth was evaluated by MTT assay (Cell Proliferation Kit I (MTT), Sigma-Aldrich), according to the manufacturer's instructions. Experiments were performed in triplicate.Apoptosis was evaluated by flow cytometry measuring annexin staining. Briefly, cells were washed once with PBS 1X and incubated for 15 minutes with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (Annexin V-FITC Apoptosis Detection Kit, Immunostep). After incubation, cells were analyzed by flow cytometry. For all samples, at least 100000 events were acquired. BD CellQuest software (BD Biosciences) was used for data analysis.

2.8. Colony growth assay

Kasumi-1 cells, transfected with pCGN-Sprouty1 and pCGN empty vector, were plated in RPMI-Soft Agar to test their clonogenic ability. Appropriate control samples were plated for each experiment. After 2 weeks, cells were stained with Crystal Violet, visualized and counted by Infinity Analyze 3 camera and processed by Lumenera software (Windows).

2.9. Statistical analysis

Statistical analyses were performed using the two-tailed Student's t-test. All the analysis with confidence level major of 95% are indicated like significant and marked as followed: * $p \leq 0,05$; ** $p \leq 0,01$; *** $p \leq 0,001$.

Peer-reviewed version available at J. Clin. Med. 2019, 8, 972; doi:10.3390/jcm8070972

3. Results

3.1. Sprouty1 mRNA and protein are both down regulated in AML patients at diagnosis

We initially analyzed the Sprouty1 gene expression by quantitative Real Time PCR (qRT-PCR) in BM and PB samples collected from 90 AML patients at diagnosis and 34 healthy subjects. Sprouty1 transcript is significantly decreased in both BM and PB of AML patients when compared to healthy subjects (Figure 1A). The median value of transcript expressed as $2^{-\Delta\Delta Ct}$ is 0.3 in BM from AML patients compared to 0.5 in BM from healthy subjects ($p \leq 0.01$) and 0.18 in PB from AML patients compared to 1.15 in PB from healthy subjects ($p \leq 0.001$). There is no significant difference in Sprouty1 gene expression according to the FAB subtypes or according to different chromosomal translocations or FLT3 mutations (data not shown). Subsequently, we investigated Sprouty1 protein amount and localization in primary leukemic cells derived from AML patients by Western blot and immunofluorescence assay. Western blot of four representative patients and one control showed the presence of the 35kDa immunoreactive protein Sprouty1 in the sample derived from healthy donor. By contrast the protein was barely detectable in leukemic cells (Figure 1B).

In line with these results, immunofluorescence assay showed that cytoplasm of normal controls were stained brightly by the anti-Sprouty1 antibody, while in AML patients the protein is completely absent (Figure 1C). To further confirm that Sprouty1 down regulatation is a specific feature of AML, we analyzed the same patients at the time of complete remission after chemotherapy. Immunofluorescence showed that the intensity and localization of Sprouty1 is completely restored as in control cells (Figure 1C).

Peer-reviewed version available at J. Clin. Med. 2019, 8, 972; doi:10.3390/jcm8070972

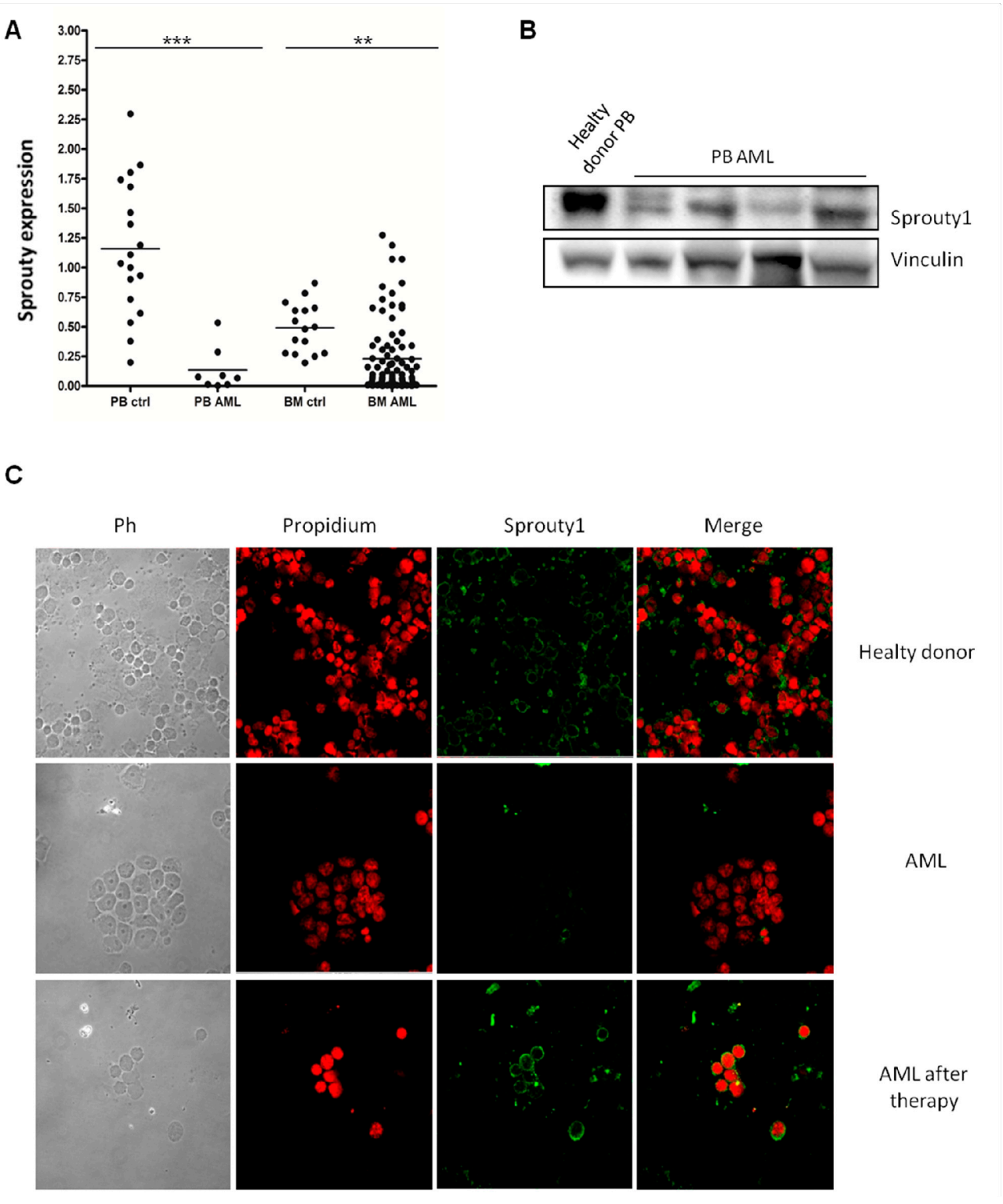

Figure 1: A) *Sprouty1* gene expression was assayed by qRT-PCR in BM and PB derived from both AML patients and normal subjects. The quantity is expressed as $2^{-\Delta\Delta Ct}$ after normalization with *Abl* housekeeping gene (** p≤0.01 and *** p≤0.001). **B)** Western blot performed with anti Sprouty1 antibody on total protein derived from PB of four representative AML cells and one PB of healthy donor. Vinculin is used as normalizer. **C)** Immunofluorescence staining assay performed on cytospun BM cells of AML or control samples. Green signal corresponds to Sprouty1 while red propidium is used to detect nuclei.

Peer-reviewed version available at J. Clin. Med. 2019, 8, 972; doi:10.3390/jcm8070972

3.2. Over expression of Sprouty1 induces apoptosis, inhibits proliferation and colonies growth in Kasumi-1 cell line

To investigate the negative role of Sprouty1 in sustaining the leukemic proliferation and favoring apoptosis defect, we transiently over expressed the Sprouty1 in Kasumi-1 cell line. After confirming the increased level of Sprouty1 protein in transfected cells (Figure 2A), we conducted proliferation and apoptosis assays. We examined the proliferation activity of transfected Kasumi-1 cells by MTT assay and we observed a significant inhibition of proliferation in cells transfected with pCGN-Sprouty1, with a 30% of reduction compared to Kasumi-1 cells transfected with control vector ($p \leq 0.01$) (Figure 2B).

Sprouty1 over expression increased significantly the number of apoptotic cells when compared to control cells, represented by cells transfected with the empty vector (mean values 18% compared to 10% respectively, $p \leq 0.05$) (Figure 2C).

Finally, we evaluated the effect of Sprouty1 on clonal growth in Kasumi-1 cells. Following transfection, cells were seeded in RPMI-Soft Agar for colony assays. Colonies growth was strongly inhibited, and size dramatically reduced compared to control cells transfected with empty vector ($p \leq 0.01$), further demonstrating the role of Sprouty1 in leukemia cell growth (Figure 2D).

Peer-reviewed version available at J. Clin. Med. 2019, 8, 972; doi:10.3390/jcm8070972

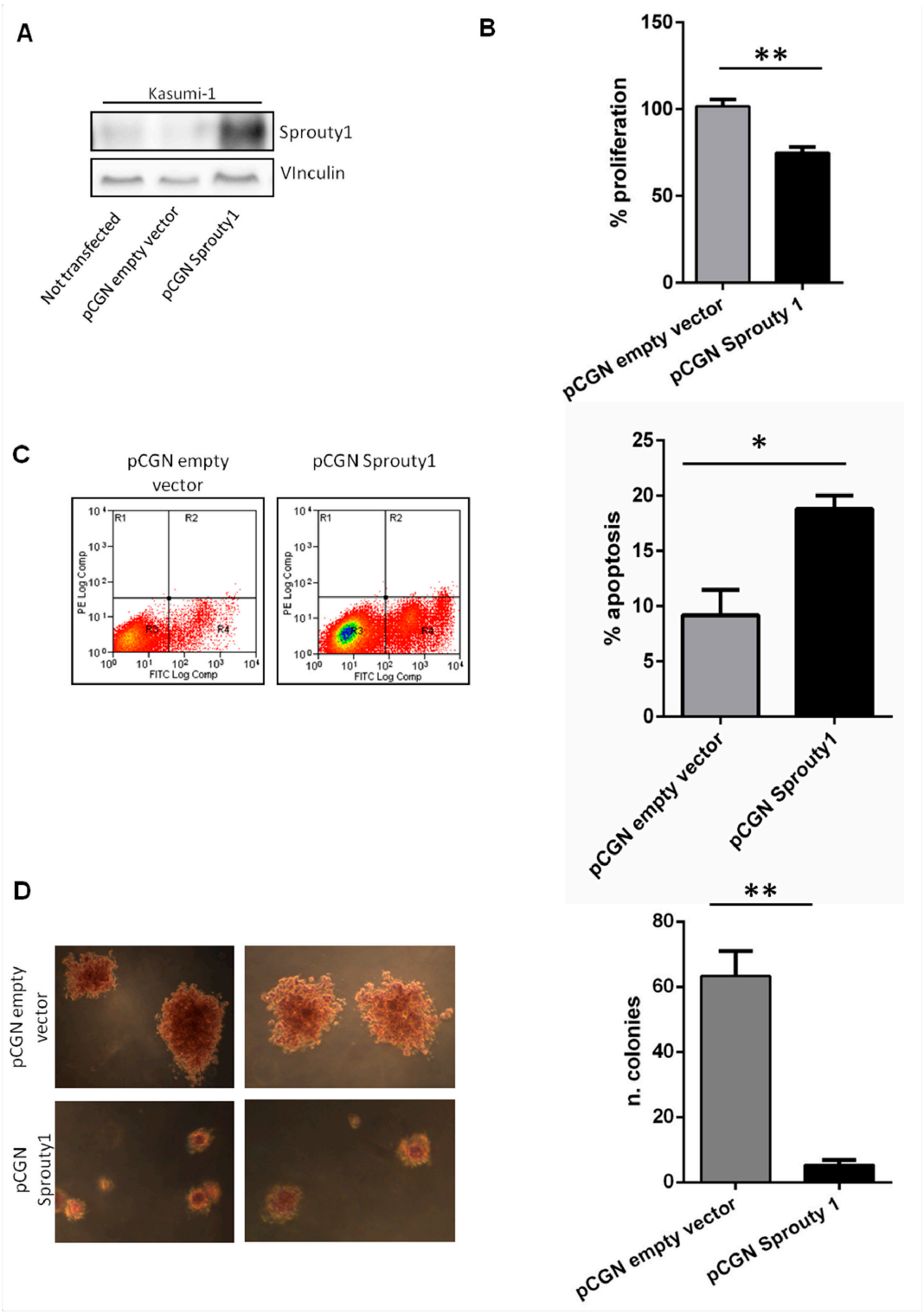

Figure 2: A) Western blot analysis and quantification performed on Kasumi-1 cell lines transfected respectively with pCGN empty vector and pCGN-Sprouty1 vector. **B)** Proliferation assay performed in Kasumi-1 cells transfected with empty or Sprouty1 vector. **C)** Apoptosis evaluated by flow cytometry after FITC Annexin-V assay on Kasumi-1 cells transfected with pCGN-Sprouty1. **D)** RPMI-Soft Agar colony assay on Kasumi-1 transfected cells . Representative colonies pictures were captured by Infinity Analyze 3 camera and processed by Lumenera software. All experiments were performed in triplicate.

Peer-reviewed version available at J. Clin. Med. 2019, 8, 972; doi:10.3390/jcm8070972

317 *3.3. FoxO3a protein is delocalized in AML patients at diagnosis*

318 In order to investigate the mechanisms leading to down regulation of Sprouty1 in AML patients, we
319 analyzed the transcription factor FoxO3a that is known to be one regulator of Sprouty family gene
320 expression [35]. Immunofluorescence assay performed on primary AML cells showed that FoxO3a is
321 exclusively localized within the cytoplasm and it is absent in the nucleus thus suggesting its
322 complete loss of the transcription activity. By contrast, FoxO3a is localized in both cytoplasm and
323 nucleus of control cells (Figure 3A). This result was confirmed in AML cells by Western blot
324 performed on cytosolic and nuclear lysates respectively. As shown in Figure 3B, a thick band is
325 observed only in the columns corresponding to cytoplasmic lysates.

326 To further assess the role of FoxO3a in down regulation of Sprouty1, we ectopically expressed
327 FoxO3a in Kasumi-1 cells. After confirming the increased protein in transfected cells (Figure 3C), we
328 evaluate if FoxO3a could positively regulate Sprouty1 by analyzing its mRNA and protein levels. As
329 shown in Figure 3C and D, both Sprouty1 protein and mRNA were significantly increased in
330 FoxO3a transfected cells, suggesting a direct cross-talk between this proteins (Figure 3E).

331

332

333

334

335

336

337

338

339

340

341

342

343

344

345

346

347

348

Peer-reviewed version available at J. Clin. Med. 2019, 8, 972; doi:10.3390/jcm8070972

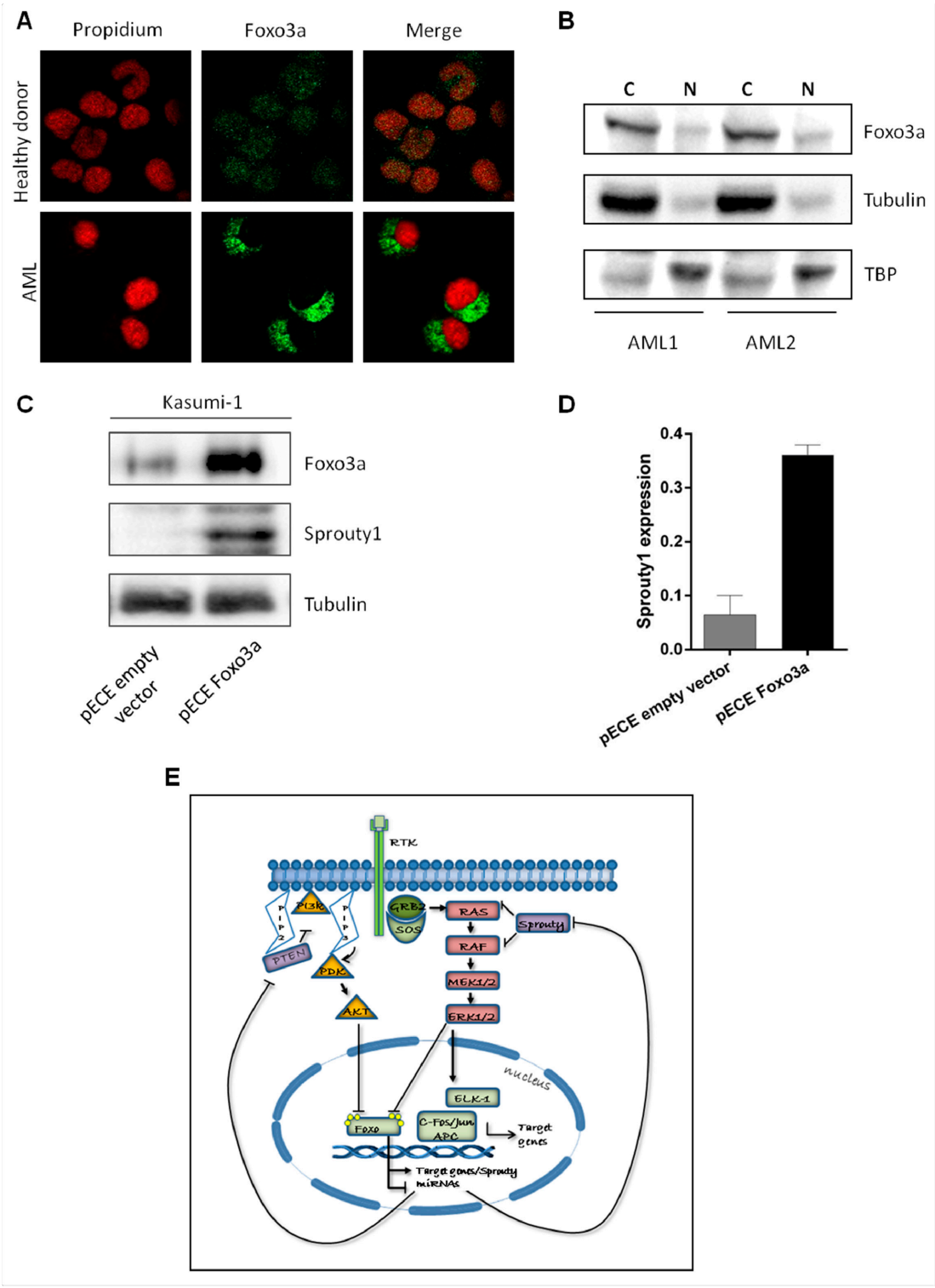

Figure 3: A) Immunofluorescence assay performed with anti-FoxO3a (green signal) on cells derived from control subjects and AML patients at diagnosis. **B)** Western blot performed with antibody against FoxO3a on lyses derived from the cytosol and nuclei of AML patients. **C)** Western blot of FoxO3a and Sprouty1 in Kasumi-1 cells transfected with FoxO3a or empty plasmids. **D)** *Sprouty1* gene expression analysis on Kasumi-1 cells transfected with FoxO3a or empty plasmids. The quantity is expressed as $2^{-\Delta\Delta Ct}$ after normalization with *Abl* housekeeping gene. **E)** Schematic representation of RAS/PI3K pathways and their negative regulation on FoxO3a.

Peer-reviewed version available at J. Clin. Med. 2019, 8, 972; doi:10.3390/jcm8070972

4. Discussion

The present study is focused on Sprouty1, an evolutionary conserved negative regulator of proliferation and cell growth mediated by RAS/MAPK signaling, in patients affected by acute myeloid leukemia. AML is a genetically heterogeneous clonal hematopoietic stem cell malignancy characterized by chromosomal abnormalities, recurrently mutated genes, epigenetic modifications affecting chromatin structure, and microRNAs deregulations [36]. RAS pathway is commonly mutated or deregulated in AML and represents a marker of leukemia progression. In this scenario, Sprouty proteins deregulation may be responsible of tumor growth, invasion and metastasis [13,37]. Germ line loss of function mutation was reported to predispose to AML [38], especially M4/M5 which have RAS pathway mostly deregulated [39,40]. Sprouty orchestrates a complex, multilayered regulatory system and mediates a crosstalk with number of effectors, mediators, and regulators of ERK pathway. Its ability to control well-characterized oncogene products suggested the expression levels of the Sprouty genes may be relevant in human carcinogenesis [2]. Down regulation of Sprouty1 has been found in carcinomas of the breast, prostate cancer, renal cell carcinoma and pediatric AML [3,19].

We demonstrated that down regulation of Sprouty1 plays a central role in sustaining the leukemic clone. Sprouty1 mRNA and protein was significantly decreased in our group of AML when compared to normal samples. Strikingly, the transcription level is restored when remission is achieved after chemotherapy. Enforcing the role of Sprouty1 in leukemia cell growth, we found that the over expression induces apoptosis, strong inhibition of proliferation and colonies growth in Kasumi-1 cell line, restoring typical defect of leukemic cells.

To investigate the mechanism of Sprouty1 down regulation, we decided to analyze FoxO3a, a regulator of Sprouty family [41,42]. In AML cells we identified a strong delocalization of FoxO3a in the cytoplasm, inactivation of its transcription activity and accumulation of the inactive phosphorylated form, as consequent of AKT phosphorylation. Furthermore, activation of ERK has been shown to phosphorylate FoxO proteins, resulting in subsequent MDM2-dependent ubiquitination and protein degradation [43]. In our samples tools, the inactivation of FoxO3a both via RAS/ERK pathway and by AKT pathway could be responsible of Sprouty1 reduction. Otherwise, post transcriptional events FoxO3a related could sustain the low Sprouty1 expression in AML, and in turn this negative feedback could imply an increase in RAS activity. In AML patients RAS and PI3K pathways are frequently deregulated or constitutively activated [4,5]. In this scenario, combined therapies using MEK and PI3K inhibitors could have synergistic effects on FOXO3a reactivation and could restore the Sprouty1 levels. An interesting approach based on the GSK2141795 has been suggested in solid tumors [44,45]. In AML a phase II trial is ongoing exploring the efficacy of this approach. (ClinicalTrials.gov Identifier: NCT01907815).

Author Contributions: VR, CP, JP designed the study, performed the experiments and wrote the manuscript. SC performed qRT-PCR experiments. MD, GA, CV provided and analyzed clinical data. GS provided final approval of the manuscript. EB and DC supervised the experiments and wrote the manuscript.

# VR and CP contributed equally to this manuscript

$ EB and DC contributed equally to this manuscript

Funding: The study was funded by grants from AIRC and MIUR (Prin-Cofin).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Gilliland, D.G.; Griffin, J.D. The roles of FLT3 in hematopoiesis and leukemia. *Blood* **2002**, *100*, 1532-1542, doi:10.1182/blood-2002-02-0492.
2. Lo, T.L.; Fong, C.W.; Yusoff, P.; McKie, A.B.; Chua, M.S.; Leung, H.Y.; Guy, G.R. Sprouty and cancer: the first terms report. *Cancer letters* **2006**, *242*, 141-150, doi:10.1016/j.canlet.2005.12.032.
3. Masoumi-Moghaddam, S.; Amini, A.; Morris, D.L. The developing story of Sprouty and cancer. *Cancer metastasis reviews* **2014**, *33*, 695-720, doi:10.1007/s10555-014-9497-1.
4. Martinez, N.; Garcia-Dominguez, C.A.; Domingo, B.; Oliva, J.L.; Zarich, N.; Sanchez, A.; Gutierrez-Eisman, S.; Llopis, J.; Rojas, J.M. Sprouty2 binds Grb2 at two different proline-rich regions, and the mechanism of ERK inhibition is independent of this interaction. *Cellular signalling* **2007**, *19*, 2277-2285, doi:10.1016/j.cellsig.2007.07.008.
5. Lao, D.H.; Chandramouli, S.; Yusoff, P.; Fong, C.W.; Saw, T.Y.; Tai, L.P.; Yu, C.Y.; Leong, H.F.; Guy, G.R. A Src homology 3-binding sequence on the C terminus of Sprouty2 is necessary for inhibition of the Ras/ERK pathway downstream of fibroblast growth factor receptor stimulation. *The Journal of biological chemistry* **2006**, *281*, 29993-30000, doi:10.1074/jbc.M604044200.
6. Hacohen, N.; Kramer, S.; Sutherland, D.; Hiromi, Y.; Krasnow, M.A. sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. *Cell* **1998**, *92*, 253-263.
7. de Maximy, A.A.; Nakatake, Y.; Moncada, S.; Itoh, N.; Thiery, J.P.; Bellusci, S. Cloning and expression pattern of a mouse homologue of drosophila sprouty in the mouse embryo. *Mechanisms of development* **1999**, *81*, 213-216.
8. Kramer, S.; Okabe, M.; Hacohen, N.; Krasnow, M.A.; Hiromi, Y. Sprouty: a common antagonist of FGF and EGF signaling pathways in Drosophila. *Development* **1999**, *126*, 2515-2525.
9. Sieglitz, F.; Matzat, T.; Yuva-Aydemir, Y.; Neuert, H.; Altenhein, B.; Klambt, C. Antagonistic feedback loops involving Rau and Sprouty in the Drosophila eye control neuronal and glial differentiation. *Science signaling* **2013**, *6*, ra96, doi:10.1126/scisignal.2004651.
10. Gross, I.; Bassit, B.; Benezra, M.; Licht, J.D. Mammalian sprouty proteins inhibit cell growth and differentiation by preventing ras activation. *The Journal of biological chemistry* **2001**, *276*, 46460-46468, doi:10.1074/jbc.M108234200.
11. Tefft, J.D.; Lee, M.; Smith, S.; Leinwand, M.; Zhao, J.; Bringas, P., Jr.; Crowe, D.L.; Warburton, D. Conserved function of mSpry-2, a murine homolog of Drosophila sprouty, which negatively modulates respiratory organogenesis. *Current biology : CB* **1999**, *9*, 219-222.
12. Yoshida, T.; Hisamoto, T.; Akiba, J.; Koga, H.; Nakamura, K.; Tokunaga, Y.; Hanada, S.; Kumemura, H.; Maeyama, M.; Harada, M., et al. Spreds, inhibitors of the Ras/ERK signal transduction, are dysregulated in human hepatocellular carcinoma and linked to the malignant phenotype of tumors. *Oncogene* **2006**, *25*, 6056-6066, doi:10.1038/sj.onc.1209635.
13. Hanafusa, H.; Torii, S.; Yasunaga, T.; Nishida, E. Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway. *Nature cell biology* **2002**, *4*, 850-858, doi:10.1038/ncb867.
14. Pasmant, E.; Gilbert-Dussardier, B.; Petit, A.; de Laval, B.; Luscan, A.; Gruber, A.; Lapillonne, H.; Deswarte, C.; Goussard, P.; Laurendeau, I., et al. SPRED1, a RAS MAPK pathway inhibitor that causes

Legius syndrome, is a tumour suppressor downregulated in paediatric acute myeloblastic leukaemia. *Oncogene* **2015**, *34*, 631-638, doi:10.1038/onc.2013.587.

15. Reich, A.; Sapir, A.; Shilo, B. Sprouty is a general inhibitor of receptor tyrosine kinase signaling. *Development* **1999**, *126*, 4139-4147.
16. Sasaki, A.; Taketomi, T.; Kato, R.; Saeki, K.; Nonami, A.; Sasaki, M.; Kuriyama, M.; Saito, N.; Shibuya, M.; Yoshimura, A. Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. *Nature cell biology* **2003**, *5*, 427-432, doi:10.1038/ncb978.
17. de Alvaro, C.; Martinez, N.; Rojas, J.M.; Lorenzo, M. Sprouty-2 overexpression in C2C12 cells confers myogenic differentiation properties in the presence of FGF2. *Molecular biology of the cell* **2005**, *16*, 4454-4461, doi:10.1091/mbc.E05-05-0419.
18. Impagnatiello, M.A.; Weitzer, S.; Gannon, G.; Compagni, A.; Cotten, M.; Christofori, G. Mammalian sprouty-1 and -2 are membrane-anchored phosphoprotein inhibitors of growth factor signaling in endothelial cells. *The Journal of cell biology* **2001**, *152*, 1087-1098.
19. Pasmant, E.; Vidaud, D.; Ballerini, P. RAS MAPK inhibitors deregulation in leukemia. *Oncoscience* **2015**, *2*, 930-931, doi:10.18632/oncoscience.274.
20. Miyamoto, K.; Miyamoto, T.; Kato, R.; Yoshimura, A.; Motoyama, N.; Suda, T. FoxO3a regulates hematopoietic homeostasis through a negative feedback pathway in conditions of stress or aging. *Blood* **2008**, *112*, 4485-4493, doi:10.1182/blood-2008-05-159848.
21. Marinkovic, D.; Zhang, X.; Yalcin, S.; Luciano, J.P.; Brugnara, C.; Huber, T.; Ghaffari, S. Foxo3 is required for the regulation of oxidative stress in erythropoiesis. *The Journal of clinical investigation* **2007**, *117*, 2133-2144, doi:10.1172/JCI31807.
22. Miyamoto, K.; Araki, K.Y.; Naka, K.; Arai, F.; Takubo, K.; Yamazaki, S.; Matsuoka, S.; Miyamoto, T.; Ito, K.; Ohmura, M., et al. Foxo3a is essential for maintenance of the hematopoietic stem cell pool. *Cell stem cell* **2007**, *1*, 101-112, doi:10.1016/j.stem.2007.02.001.
23. Kaestner, K.H.; Knochel, W.; Martinez, D.E. Unified nomenclature for the winged helix/forkhead transcription factors. *Genes & development* **2000**, *14*, 142-146.
24. Dijkers, P.F.; Medema, R.H.; Lammers, J.W.; Koenderman, L.; Coffer, P.J. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Current biology : CB* **2000**, *10*, 1201-1204.
25. Furukawa-Hibi, Y.; Yoshida-Araki, K.; Ohta, T.; Ikeda, K.; Motoyama, N. FOXO forkhead transcription factors induce G(2)-M checkpoint in response to oxidative stress. *The Journal of biological chemistry* **2002**, *277*, 26729-26732, doi:10.1074/jbc.C200256200.
26. Medema, R.H.; Kops, G.J.; Bos, J.L.; Burgering, B.M. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* **2000**, *404*, 782-787, doi:10.1038/35008115.
27. Tran, H.; Brunet, A.; Grenier, J.M.; Datta, S.R.; Fornace, A.J., Jr.; DiStefano, P.S.; Chiang, L.W.; Greenberg, M.E. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. *Science* **2002**, *296*, 530-534, doi:10.1126/science.1068712.
28. Hribal, M.L.; Nakae, J.; Kitamura, T.; Shutter, J.R.; Accili, D. Regulation of insulin-like growth factor-dependent myoblast differentiation by Foxo forkhead transcription factors. *The Journal of cell biology* **2003**, *162*, 535-541, doi:10.1083/jcb.200212107.

29. Brunet, A.; Bonni, A.; Zigmond, M.J.; Lin, M.Z.; Juo, P.; Hu, L.S.; Anderson, M.J.; Arden, K.C.; Blenis, J.; Greenberg, M.E. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **1999**, *96*, 857-868.
30. Dijkers, P.F.; Birkenkamp, K.U.; Lam, E.W.; Thomas, N.S.; Lammers, J.W.; Koenderman, L.; Coffer, P.J. FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. *The Journal of cell biology* **2002**, *156*, 531-542, doi:10.1083/jcb.200108084.
31. Kaestner, K.H. The hepatocyte nuclear factor 3 (HNF3 or FOXA) family in metabolism. *Trends in endocrinology and metabolism: TEM* **2000**, *11*, 281-285.
32. Tothova, Z.; Gilliland, D.G. FoxO transcription factors and stem cell homeostasis: insights from the hematopoietic system. *Cell stem cell* **2007**, *1*, 140-152, doi:10.1016/j.stem.2007.07.017.
33. Hu, M.C.; Lee, D.F.; Xia, W.; Golfman, L.S.; Ou-Yang, F.; Yang, J.Y.; Zou, Y.; Bao, S.; Hanada, N.; Saso, H., et al. IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. *Cell* **2004**, *117*, 225-237.
34. Kornblau, S.M.; Singh, N.; Qiu, Y.; Chen, W.; Zhang, N.; Coombes, K.R. Highly phosphorylated FOXO3A is an adverse prognostic factor in acute myeloid leukemia. *Clinical cancer research : an official journal of the American Association for Cancer Research* **2010**, *16*, 1865-1874, doi:10.1158/1078-0432.CCR-09-2551.
35. Melnik, B.C. MiR-21: an environmental driver of malignant melanoma? *Journal of translational medicine* **2015**, *13*, 202, doi:10.1186/s12967-015-0570-5.
36. Prokocimer, M.; Molchadsky, A.; Rotter, V. Dysfunctional diversity of p53 proteins in adult acute myeloid leukemia: projections on diagnostic workup and therapy. *Blood* **2017**, *130*, 699-712, doi:10.1182/blood-2017-02-763086.
37. Kato, R.; Nonami, A.; Taketomi, T.; Wakioka, T.; Kuroiwa, A.; Matsuda, Y.; Yoshimura, A. Molecular cloning of mammalian Spred-3 which suppresses tyrosine kinase-mediated Erk activation. *Biochemical and biophysical research communications* **2003**, *302*, 767-772.
38. Pasmant, E.; Ballerini, P.; Lapillonne, H.; Perot, C.; Vidaud, D.; Leverger, G.; Landman-Parker, J. SPRED1 disorder and predisposition to leukemia in children. *Blood* **2009**, *114*, 1131, doi:10.1182/blood-2009-04-218503.
39. Sano, H.; Shimada, A.; Taki, T.; Murata, C.; Park, M.J.; Sotomatsu, M.; Tabuchi, K.; Tawa, A.; Kobayashi, R.; Horibe, K., et al. RAS mutations are frequent in FAB type M4 and M5 of acute myeloid leukemia, and related to late relapse: a study of the Japanese Childhood AML Cooperative Study Group. *International journal of hematology* **2012**, *95*, 509-515, doi:10.1007/s12185-012-1033-x.
40. Renneville, A.; Roumier, C.; Biggio, V.; Nibourel, O.; Boissel, N.; Fenaux, P.; Preudhomme, C. Cooperating gene mutations in acute myeloid leukemia: a review of the literature. *Leukemia* **2008**, *22*, 915-931, doi:10.1038/leu.2008.19.
41. Cabrita, M.A.; Christofori, G. Sprouty proteins, masterminds of receptor tyrosine kinase signaling. *Angiogenesis* **2008**, *11*, 53-62, doi:10.1007/s10456-008-9089-1.
42. Nakae, J.; Oki, M.; Cao, Y. The FoxO transcription factors and metabolic regulation. *FEBS letters* **2008**, *582*, 54-67, doi:10.1016/j.febslet.2007.11.025.

43. Yang, J.Y.; Zong, C.S.; Xia, W.; Yamaguchi, H.; Ding, Q.; Xie, X.; Lang, J.Y.; Lai, C.C.; Chang, C.J.; Huang, W.C., et al. ERK promotes tumorigenesis by inhibiting FOXO3a via MDM2-mediated degradation. *Nature cell biology* **2008**, *10*, 138-148, doi:10.1038/ncb1676.

44. Jokinen, E.; Koivunen, J.P. MEK and PI3K inhibition in solid tumors: rationale and evidence to date. *Therapeutic advances in medical oncology* **2015**, *7*, 170-180, doi:10.1177/1758834015571111.

45. Roy, S.K.; Srivastava, R.K.; Shankar, S. Inhibition of PI3K/AKT and MAPK/ERK pathways causes activation of FOXO transcription factor, leading to cell cycle arrest and apoptosis in pancreatic cancer. *Journal of molecular signaling* **2010**, *5*, 10, doi:10.1186/1750-2187-5-10.