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Vav1 up-modulates the tumor suppressor miR-29b in cells from acute myeloid leukemia (AML) and triple negative breast cancer (TNBC)

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Introduction

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1.1 Vav family

The Vav family has currently five known members, three in mammalian cells (Vav1, Vav2, and Vav3) and two in invertebrates (*C. elegans* Vav and *D. melanogaster* Vav). Despite all genes of the family are expressed in hematopoietic cells, the transcripts of Vav1 are present in blood cells in greater quantities than those of Vav2 and Vav3 (Tybulewicz, 2005; Bustelo, 2002).

All Vav proteins have similar structures (Figure 1) and sequence analysis showed that they contain a Dbl homology (DH) region, typical of the GDP/GTP exchange factors (GEFs), which activate Rho-family GTPases (Adams, 1992; Zheng, 2001). This domain is flanked by several domains thought to regulate the GEF activity. Amino-terminal to the DH domain is a calponin homology (CH) domain and an acidic domain, whereas to the carboxy-terminal side is a pleckstrin homology (PH) and a C1 domain.

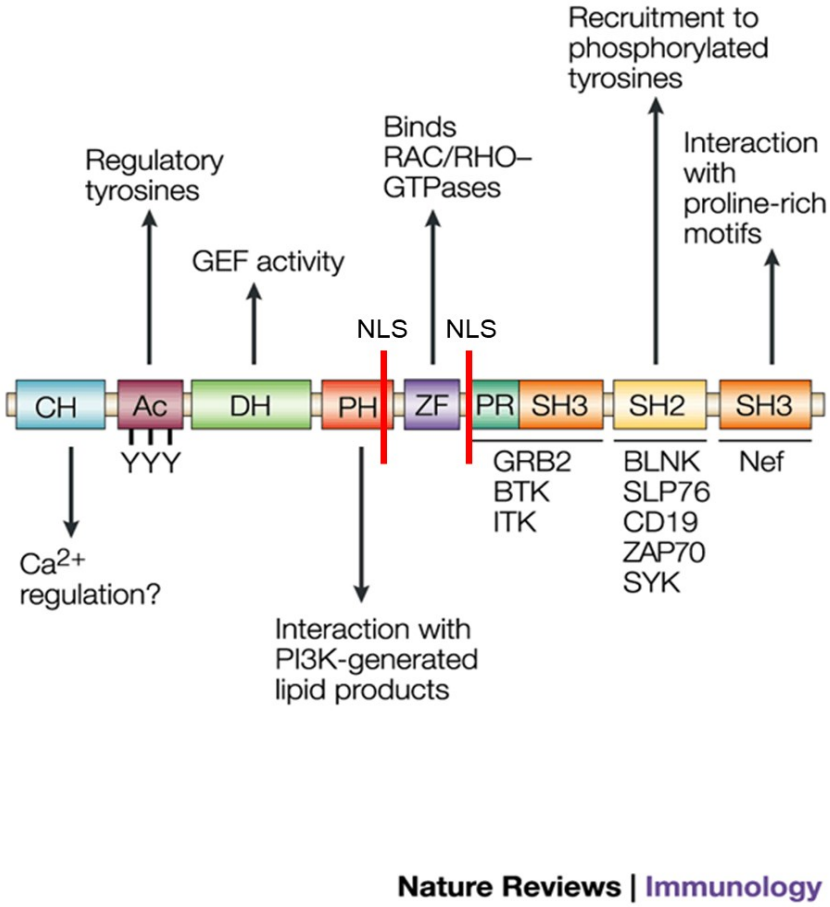

Figure 1. Structural domains of Vav proteins (Turner and Billadeau, 2002).

At the carboxy-terminal end of the protein there are two SH3 domains and one SH2 domain, which are most likely to be involved in protein–protein interactions (Tybulewicz, 2005). Finally, the presence of two nuclear localization sequences (NLS) justifies the localization of Vav1 inside the nuclear compartment (Figure 1) (Katzav, 2007).
Through its domains Vav participates in various cellular responses including actin cytoskeleton reorganization, gene transcription, and development and activation of immune cells (Katzav, 2015).

1.2 Vav function: GEF activity

Biochemical analysis confirmed that the DH domain of Vav confers to the proteins GEF activity, although there has been disagreement with respect to the specific GTPases these proteins can activate. In some reports it has been demonstrated that Vav1 is a GEF for Rac1, Rac2 and RhoG, that Vav2 is a GEF for RhoA, RhoB and RhoG, whereas Vav3 preferentially activates RhoA, RhoG and, to a lesser extent, Rac1 (Bustelo, 1992; Crespo, 1997; Schuebel, 1998). In other reports it has been shown that Vav1 may also act on RhoA and CDC42, and that Vav2 may activate Rac1 and CDC42 (Han 1997; Abe, 2000).

The DH domain showed the typical structure with 11 alpha helices forming a flattened, elongated bundle (Zhang, 1995). The stabilization of this structure was due to the hydrophobic interaction of three residues of the N-terminal alpha helix (I173, Y174, L177) with residues present in the Vav DH region (Y209, T212, P320, L325, V328). As a consequence of this folding, the N-terminal alpha helix made the DH domain inaccessible to the GTPase substrate. This structure immediately demonstrated the mechanism of Vav inhibition and, as a bonus, suggested that perhaps the tyrosine residue involved in those interactions was the key one in the activation of Vav by tyrosine phosphorylation. The structure of the Vav DH domain was unchanged when the folding of the phosphorylated and non-phosphorylated forms of Vav were compared. However, the phosphorylation of Y174 induced the release of the N-terminal inhibitory alpha helix from the GTPase binding site. Due to this new conformation, the Vav protein acquired an “open” configuration capable of making stable interactions with Rac1. The residue located at position 174 of Vav and its surrounding amino acid sequences are conserved in the three mammalian Vav members and in *D. melanogaster* Vav, suggesting that this regulatory mechanism is taking place in most Vav family members. (Bustelo 2002; Katzav, 2015) (Figure 2).

The level of activity of Vav proteins seems also dependent on the type of lipids bound to their PH domains, as firstly demonstrated by Broek and coworkers (Han, 1998). Using *in*

vitro exchange reactions, this group showed that the enzyme activity of phosphorylated Vav could be increased approximately two fold when exchange reactions were conducted in the presence of PI-3,4 P_2 or PIP_3, two products of phosphatidylinositol-3 kinase (PI3K). Conversely, the GDP/GTP exchange activity of Vav was totally inhibited when the PI3K substrate PI-4-5-P_2 (PIP_2) was included in the reactions. These effects are due to the specific binding of phospholipids to the PH domain of Vav. Based on these results, it was proposed that the PIP_2-bound to PH domain of Vav inhibits its catalytic activity due to the establishment of intramolecular interactions with other regions. Such intramolecular interaction resulted in the inability of Vav to undergo optimal tyrosine phosphorylation and, as a consequence, precluded the optimal stimulation of Vav exchange activity (Bustelo, 2002).

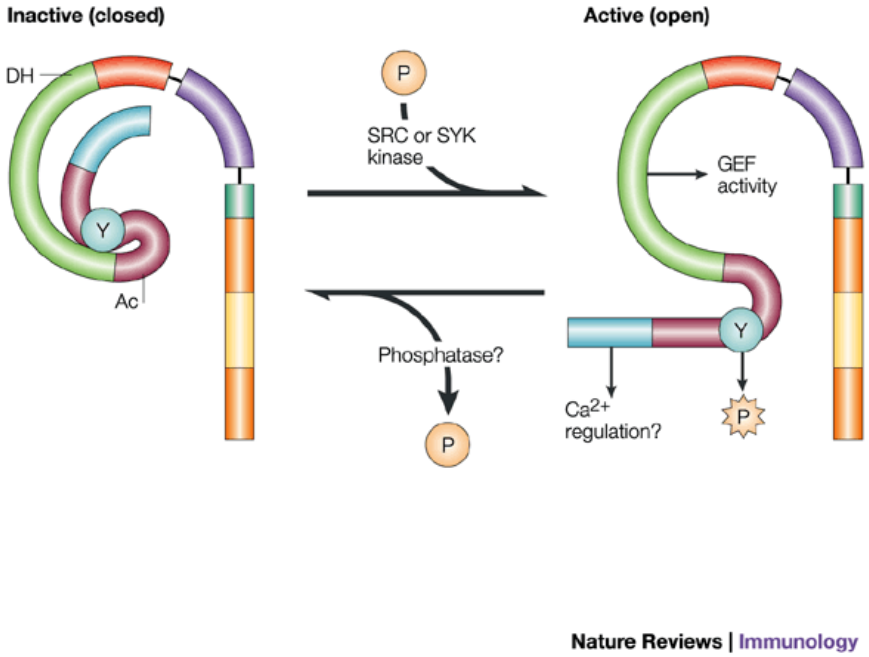

Figure 2. Hypothetical mechanism of activation of Vav-family proteins. In the inactive state, Vav is folded in such a way as to inhibit the GEF activity of the DH domain (Turner M. and Billadeau D., 2002).

In immune cells, endogenous Vav1 is tyrosine phosphorylated following activation of many receptors, including the T-cell receptor (TCR) (Margolis, 1992; Bustelo, 1992), B-cell receptor (BCR) (Bustelo, 1992), FcRI (Manetz, 2001), cytokine receptors (Evans, 1993), NK receptors (Galandrini, 1999), chemokine receptors (Garcia-Bernal, 2005) and integrins (Zheng, 1996). The activation of Vav1 by these receptors leads to different outcomes depending on the specific hematopoietic cell type (Katzav, 2015).

1.3 Vav1 as a scaffold protein

Many of Vav1 functions are exerted via its ability to associate with other proteins (Bustelo, 2000; Katzav, 2004; Tybulewicz, 2005; Katzav, 2009). For instance, several proteins associate with the CH region, including Socs1, a downstream component of the Kit receptor tyrosine kinase signaling pathway (De Sepulveda, 2000); ENX-1, a putative transcriptional regulator of homeobox gene expression (Hobert, 1996); Ly-GDI, a regulator of Rho GTPases (Groysman, 2000); and calmodulin (Zhou, 2007).

In both lymphoid and myeloid cells, like other proteins with a GEF activity, Vav1 mediates a number of cytoskeletal-associated cellular processes, including the formation of immunological synapse and phagocytosis of T cells (Reynolds, 2004) and chemotaxis of neutrophils and macrophages (Kim, 2003; Gakidis, 2004; Vedham, 2005), all requiring profound modification of actin organization. On the other hand, Vav1 may mediate actin reorganization through other, GEF-independent mechanisms, based on the presence in its structure of a number of tyrosines and domains potentially involved in protein–protein interactions. A role of Vav1 in actin polymerization as an adapter protein that links signaling and cytoskeletal molecules has been described in T cells, in which Vav1 binds constitutively Talin and Vinculin, anchoring the actin cytoskeleton to the plasma membrane. Also the interaction of Vav1 with the cytoskeletal protein Zyxin and Dynamin 2 has been demonstrated in the same cell model (Hornstein, 2004; Gomez, 2005).

The SH2 domain of Vav1 interacts with auto-phosphorylated tyrosine kinases such as ZAP-70 (Katzav, 1994) and Syk (Darby, 1994) and with the adapter proteins SLP76 (Wu, 1996) and Blnk (Johmura, 2003). Vav1's N-terminal SH3 domain binds the adapter protein Grb2 (Ye, 1994) shown to be necessary for translocation of Vav1 to the plasma membrane and its interaction with upstream tyrosine kinases in lymphoid cells (Katzav, 2015).

In lymphoid cells, it has been reported that Vav1 and CD28 can synergize for the gene transcription for IL-2. Furthermore, CD28 binds the Grb-2 protein and is likely to be such bridge fencing complex for Vav1. The inability of Grb-2 to bind CD28 prevents Grb-2 itself to cooperate with Vav1 in the up-regulation of the Nuclear Factor of Activated T cells (NFAT)/Activator Protein-1 (AP-1) complex activity (Schneider, 2008).

Via its carboxy-terminal SH3, Vav1 is able to form complexes with a wide variety of proteins including cytoskeletal regulators (Zyxin) (Hobert, 1996), RNA-binding proteins (hnRNP-K, hnRNP-C and Sam68) (Hobert, 1994; Lazer, 2007), transcriptional modulators

including NFAT, AP-1, Nuclear Factor κB (NF-κB) (Costello, 1999; Wu, 1995), ubiquitination factors, viral proteins, a Kruppel-like protein, and Dynamin 2 (Bustelo, 2000; Turner, 2002; Tybulewicz, 2005). While the consequences of all these interactions are not yet known, Vav1's ability to interact with many proteins likely allows it to function in multiple signaling pathways (Katzav, 2015).

As previously mentioned, two nuclear localization sequences (NLS) were identified in the Vav1 protein and a number of studies have shown the presence of Vav1 in the nucleus of numerous hematopoietic cell types, both normal and derived from leukemia. In particular, nuclear Vav1 was demonstrated in T lymphocytes and Jurkat cells, HL-60 and NB4 promyelocytes, megakaryoblasts UT7 (Margolis, 1992; Romero, 1996; Romero, 1998; Houlard, 2002; Bertagnolo, 2005; Gomez, 2005).

2. Vav1 in myeloid leukemia

Vav1 is specifically expressed in hematopoietic tissues, and is generally down-regulated in both lymphoid and myeloid leukemia (Katzav, 2015).
Several experimental evidences indicate an involvement of Vav1 in the transduction of signal of cytokines or hematopoietic growth factors in human tumor myeloid precursors. In particular, phosphorylation of Vav proteins has been reported in a wide range of cell types and downstream of many different receptors, including the B-cell antigen receptor, FceRI,FcgRI/II/III, growth factor receptors, integrins, cytokine receptors and chemokine receptors (Bustelo, 2000; Tybulewicz, 2005). Concerning myeloid leukemia, my research group identified for Vav1 a crucial role during the agonist induced maturation of cells derived from acute promyelocytic leukemia (APL), the M3 subtype of acute myeloid leukemia.

2.1 Vav1 promotes the neutrophil-like maturation of APL-derived cells.

ATRA-based therapy represents, until today, the standard cure of APL patients and ATRA treatment of APL constitutes, at present, the only example of successful differentiation therapy of a human cancer, in which tumor cells are induced to complete their maturation to neutrophils (Bertagnolo, 2012). Acute promyelocytic leukemia (APL) was characterized by abnormal promyelocytes showing a translocation involving chromosome 17 and 15 and resulting in the PML-RARα fusion protein which was demonstrated to be involved in the tumorigenesis of APL (He, 2018). ATRA administration is able to induce the release of the PML–RARα transcriptional repression and the degradation of the fusion protein, ended to

promote maturation/apoptosis of tumoral promyelocytes. Neutrophil-like differentiation includes characteristic changes of cell morphology which occur in a coordinated sequence leading to acquisition of a highly typical mature phenotype. These architectural changes occur dramatically in the nucleus, making indeed the modifications of the nuclear shape, in addition to the expression of specific pattern of surface antigens, an easy-to-follow marker of neutrophil maturation (Sanchez, 1999; Bertagnolo, 2004).

APL-derived promyelocytes contain levels of Vav1 variably lower than those found in mature neutrophils. Treatments with differentiating doses of ATRA induce a significant increase of Vav1 expression in both cytoplasm and nuclear compartment of primary blasts from APL patients and APL-derived cell lines (Bertagnolo, 2005). The issue of whether the increase of Vav1 observed in differentiation of tumoral promyelocytes is merely designed to the function of the protein in mature cells or, more intriguingly, it is functionally relevant to the maturation mechanism, has been addressed by studies in which the expression of Vav1 was forcedly modulated in HL-60 and NB4 cells, APL-derived cell lines which constitute reference cell models since they are blocked at different stages of granulocytic differentiation and reach different levels of neutrophil maturation (Breitman, 1980; Lanotte, 1991). These experiments unequivocally demonstrate that Vav1 is not dispensable for the progression of tumoral promyelocytes along the granulocytic lineage and supports the role of ATRA in regulating the maturation process, in terms of both surface antigens expression and modifications of cell/nucleus morphology (Bertagnolo, 2005; Bertagnolo, 2008; Bertagnolo, 2012).

In ATRA-treated HL-60 cells, the mechanism by means of which tyrosine-phosphorylated Vav1 regulates actin cytoskeleton implies the interaction of Vav1 with the p85 regulatory subunit of PI3K. Studies aimed to establish the functional significance of this interaction have demonstrated that, in maturating myeloid precursors, PI3K activity closely depends on its association with Vav1 tyrosine phosphorylated by Syk and that when Vav1/PI3K interaction and/or PI3K activity are abrogated, the phenotypic differentiation of ATRA-treated HL-60 is compromised, in terms of both surface antigen expression and modifications of cellular/nuclear morphology, (Bertagnolo, 1999; Bertagnolo, 2004). Also actin is present in the ATRA-induced protein complexes containing Vav1 and PI3K in HL-60 cells. Remarkably, when the Vav1/PI3K association is impaired by down-modulation of Syk activity, the formation of PI3K/actin complexes is reduced (Bertagnolo, 2004), suggesting that the interaction of PI3K with tyrosine-phosphorylated Vav1 is essential for its association with actin. Since the recovery of 3-phosphoinositides is strongly reduced

when the Vav1-dependent interaction between PI3K and actin is abrogated, it can be concluded that Vav1 regulates the physical contact of PI3K with its cytoskeleton-associated substrates. These evidences assigned to Vav1/PI3K interaction a prominent role in the regulation of cytoskeleton, alternative to the described function of 3-phosphoinositides in modulating GEF activity of Vav1 (Han, 1998) and indicated that, in addition to play a regulatory role in Vav1 activation, PI3K activity may itself be regulated by Vav1

2.1.1 Vav1 regulates gene expression

In myeloid and lymphoid cells, Vav1 seems to be involved in regulating DNA transcription by direct interaction with, or as a facilitator of, transcription factors. In particular, Vav1 regulates NFAT, AP-1 and NF-κB in T-cells in response to TCR stimulation, and exerts a specific role in regulating the CREB-dependent gene transcription (Katzav, 2004; Schneider, 2008). Direct evidence for the presence of Vav1 in active transcriptional complexes with NFAT and NF-kB-like has been demonstrated in mast cells, in which Vav1 acts as a facilitator of transcriptional activity (Houlard, 2002).

As a consequence of ATRA administration, Vav1 tyrosine-phosphorylated by Syk accumulates inside the nuclear compartment of APL-derived cells and becomes involved in the reorganization of their nuclear architecture. These structural changes are at the basis of both transcription and post-transcriptional events, suggesting indeed that Vav1 may play a prominent role also in regulating ATRA-related gene expression. The silencing of Vav1 expression during ATRA administration has unambiguously allowed to ascertain its role in regulating ATRA-dependent gene expression, further confirming that the up-regulation of Vav1 is not only a maturation-related phenomenon but is also a key event in granulocytic differentiation of tumoral myeloid precursors.

In APL-derived cells, nuclear Vav1 associates with PU.1 (Brugnoli, 2010), a transcription factor induced by ATRA and with a crucial role in the completion of granulocytic differentiation of tumoral myeloid precursors (Mueller, 2006). On the other hand, in NB4 cells, like in other tumoral myeloid precursors (Denkinger, 2002), PU.1 regulates the expression of Vav1 induced by ATRA (Brugnoli, 2010).

In tumoral myeloid precursors, PU.l is a major determinant of the myeloid expression of CD11b (Pahl, 1993; Kastner, 2008), an integrin receptor whose surface expression increases during differentiation of APL-derived cell lines (Barber, 2008). The over-expression of PU.1 might influence the phenotype and restore differentiation of primary

myeloid leukemic blasts (Durual, 2007), and its silencing counteracts the ATRA ability to induce the expression of the granulocytic marker CD11b (Mueller, 2006). In NB4 cells treated with ATRA, PU.1 is recruited to its consensus sequence within the CD11b promoter and may be used by ATRA to promote CD11b expression during the late stages of the maturation of APL-derived cells. Although unphosphorylated Vav1 is also recruited to the PU.1 consensus sequence on the CD11b promoter in untreated NB4 cells, the participation of Vav1 to molecular complexes including PU.1 has been ruled out. ATRA treatment, by inducing an increase in Syk-dependent tyrosine phosphorylation of Vav1, displaces Vav1 from existing molecular complexes on the CD11b promoter, an event that seems to promote the formation of PU.1-containing complexes. In fact, when the amount of Vav1 is forcedly reduced or its tyrosine phosphorylation is inhibited during the differentiation treatment, the formation of a PU.1-containing complex is compromised (Brugnoli, 2010). It is then conceivable that Vav1, and in particular Vav1 tyrosine-phosphorylated by Syk, regulates the recruitment of PU.1 to its consensus sequence on the CD11b promoter region and, possibly, the expression of this surface antigen (Figure 3) (Bertagnolo, 2012).

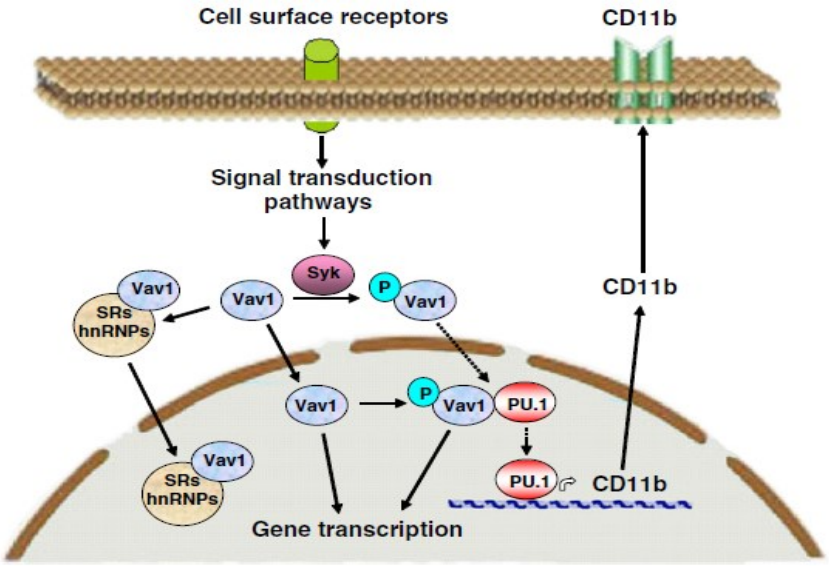

Figure 3. Schematic representation of Vav1 as a molecule able to regulate different aspects of ATRA-dependent gene expression. In APL-derived cells, Vav1 is involved in regulating the ATRA-dependent gene transcription, in terms of both mRNA and protein expression. In particular, Vav1 tyrosine-phosphorylated by Syk is part of molecular complexes with the transcription factor PU.1 and may be responsible of regulating the recruitment of PU.1 to its consensus sequence on the CD11b promoter. In addition, Vav1 may participate to RNA processing by carrying into the nucleus molecules involved in modulating mRNA production and stability, like SR and hnRNPs proteins (Bertagnolo, 2012).

In APL-derived cells treated with ATRA, Vav1 might regulated protein expression also acting at post-transcriptional level. In NB4 cells treated with ATRA, the activation of RARα-mediated gene transcription (Lee, 2002) and the regulation of posttranscriptional events (Harris, 2004) result in profound modulations of the nuclear protein pools and are accompanied by fundamental changes in nuclear architecture and activity. Experiments in which the expression of Vav1 was down-modulated during ATRA treatment of NB4 cells demonstrated that Vav1 regulates the nuclear amount and/or the mRNA levels of ATRA-modulated proteins (Bertagnolo, 2011). In particular, the response to ATRA involves, inside the nuclear compartment, a Vav1-dependent modulation of hnRNP D, hnRNP K and hnRNP H3. hnRNPs are RNA-binding proteins with important roles in multiple aspects of nucleic acid metabolism (Han SP, 2010) and variously implicated in cancer development (Carpenter, 2004). Since some hnRNP proteins continuously shuttle between the nucleus and the cytoplasm (Hobert, 1994; Bustelo, 1995), Vav1 may act as a scaffold protein specifically involved in regulating the redistribution between cytoplasm and nuclear compartment of proteins with specific roles in RNA processing (Figure 3) (Bertagnolo; 2012).

2.1.2 Vav1 regulates miRNAs expression

In both normal and malignant myelopoiesis, the activity of PU.1 is considered to be crucial, since it regulates the expression of growth factor receptors, adhesion molecules, mediators of intracellular signaling cascades and nuclear proteins, including Vav1 (Burda, 2010; Denkinger, 2002; Kastner, 2008), and various miRNAs, some of which have been related to myeloid leukemogenesis (Alemdehy, 2012; De Marchis, 2009; Sun, 2013). Among the latter, miR-142-3p has been found to be deregulated in more than 90 % of all myeloid leukemias, including APL (Wang F, 2012), and its up-regulation has been reported to be characteristic for agonist-induced monocytic and granulocytic differentiation of AML-derived cells (Wang XS, 2012), suggesting a possible role as target in the treatment of AML. Stemming from the data indicating that in a murine model of myeloid leukemia PU.1 plays a critical role in initiating miR-142 expression (Wang XS, 2012), my research group demonstrated that the expression of miR-142-3p in differentiating APL-derived NB4 cells is also dependent on PU.1. They also showed that Vav1 is essential for the recruitment of this transcription factor to its cis-binding element on the miR-142 promoter. In addition, they found that, in ATRA-treated NB4 cells, miR-142-3p sustains the agonist-induced increases in both PU.1 and Vav1. In conclusion, a Vav1/PU.1/miR-142-3p network sustains the ATRA-induced granulocytic differentiation of promyelocytic

cells, revealing the contribution of Vav1 in regulating the expression of specific miRNAs (Figure 4) (Grassilli, 2016).

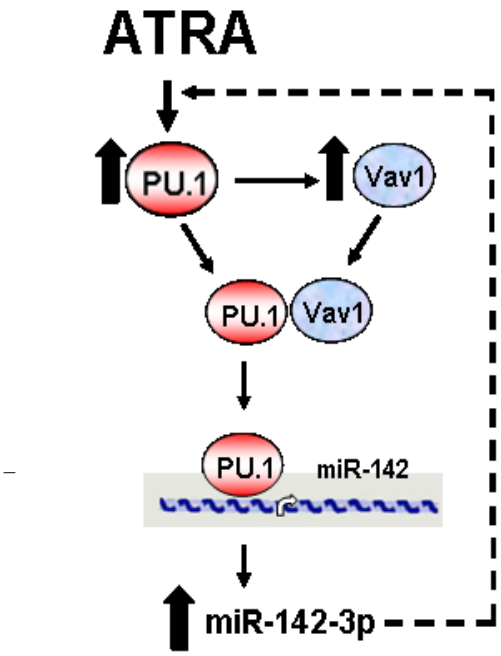

Figure 4. Schematic representation of the PU.1/Vav1/miR-142-3p network induced by ATRA in NB4 cells (Grassilli, 2016).

2.2 Vav1 and monocytic/macrophagic differentiation of AML-derived cells

HL-60 and NB4 cell lines can be differentiated along the monocytes/macrophages lineage by PMA, a stable analogue of 2,3-diacyloglycerol (Jasek, 2008; Murao, 1983; Song, 1998). Both expression and tyrosine phosphorylation of Vav1 increase during the PMA-induced acquisition of a monocyte-like phenotype of HL-60 and NB4 cells (Bertagnolo, 2011), consistently with the notion that mature monocytes express Vav1 and that proper amounts of the protein are necessary for their inflammation-related functions (Hall, 2006; Bhavsar, 2009). On the other hand, and in contrast to what observed in the ATRA-treatment of the same cell line, Syk is not recruited by PMA (Bertagnolo, 2011), consistent with the notion that, at least in HL-60 cells, Syk might exert a narrower role, restricted to directing cells toward granulocyte differentiation (Qin, 1997). In both HL-60 and NB4 cells, PMA induces a relevant increase of Vav1 phosphorylation on Tyr174 (Bertagnolo, 2011), even if a role as a GEF was not demonstrated for Vav1 in this model of cell differentiation.

By silencing the expression of Vav1 induced by PMA, a crucial role for Vav1 has been demonstrated also in differentiation of APL-derived cells to monocytes/macrophages

(Bertagnolo, 2011). In particular, also in this cell model, Vav1 regulates the expression of the CD11b surface antigen, which is induced by PMA and constitutes a marker for monocyte differentiation. Also cell adhesion is affected by down-modulation of Vav1 during PMA treatment of HL-60 and NB4 cells, in agreement with the data obtained on macrophages from Vav1-/- mice, which show a smaller adhesive area or decreased adhesion efficiency (Wells, 2005). In PMA-treated NB4 cells, at variance to what observed during granulocytic differentiation, down-modulation of Vav1 does not affect expression and architectural organization of α-tubulin (Bertagnolo, 2011), indicating that, during the maturation process of APL-derived cells, Vav1 exerts an agonist- and lineage-specific role in regulating this microtubule component. In HL-60 and NB4 cells treated with PMA, Vav1 regulates actin expression (Bertagnolo, 2011), further confirming that Vav1, besides to be involved in the formation of filaments, takes part to cytoskeleton reorganization also as a modulator of protein expression.

3. Vav1 in breast tumors

3.1 Vav1 nuclear localization predicts favorable outcome

As we above reported, the multidomain protein Vav1 is physiologically expressed only in the hematopoietic system in which it participates to cytoskeleton reorganization and gene transcription (Romero and Fischer, 1996; Tybulewicz, 2005). In recent years, aberrant expression of Vav1 has been reported in non-hematopoietic cancers (Hornstein, 2003; Fernandez-Zapico, 2005; Bartolome, 2006; Lazer, 2009; Wakahashi, 2013; Qi, 2015; Zhu, 2017), in which this protein is involved in signal transduction processes correlated with tumor phenotype (Fernandez-Zapico, 2005; Qi, 2015; Zhu, 2017).

Vav1 is expressed in the majority of breast carcinomas (Sebban, 2013), in which we have previously demonstrated a peculiar localization inside the nucleus of tumor cells (Grassilli, 2014). High amounts of nuclear Vav1 are positively correlated with low incidence of relapse, regardless phenotype and molecular subtype of breast neoplasia. In particular, Kaplan-Meier plots showed an elevated risk of distant metastasis in patients with low Vav1 expression compared with patients with high Vav1 expression in their tumors. Experiments performed with breast tumor-derived cells indicated that Vav1 negatively modulates their invasiveness *in vitro* and their metastatic efficiency *in vivo*, possibly by affecting the expression of genes involved in invasion and/or metastasis of breast tumors (Grassilli, 2014).

Still largely unknown and controversial is the requirement of an epithelial to mesenchymal transition (EMT)-like process for metastasis of solid tumors (Chui, 2013). The role of Vav1 in modulating EMT has been reported in cells derived from ovarian cancer in which, at variance with breast tumor, the expression of the protein correlates with a poor prognosis of early stage patients (Wakahashi, 2013). In breast tumor-derived cells, we failed to show significant correlation between Vav1 and the epithelial marker E-cadherin as well as of the mesenchymal protein Vimentin. At variance, when we down-modulated the expression of Vav1 in low invasive breast tumor-derived BT-474 cells, we found the up-regulation of genes encoding for transcription factors known to be activated during breast tumor metastasis (CTNNB1, GSC and TWIST1), and for AHNAK and Akt1, variously involved in tumorigenesis and cell motility. On the other hand, the down-modulated genes codify for SNAI2, B2M and TFPI2, proteins with a tumor suppressor role in breast cancer. In Vav1-silenced MDA-MB-231 cells, with the exception of SNAI3, all the up-regulated genes are involved in breast tumor malignancy and include FOXC2, PDGFRB and members of the WNT family, whose products are known to sustain the metastatic process of breast tumors.

These data suggest a possible role of Vav1 in modulating EMT in breast cancer and indicate that the evaluation of nuclear Vav1 levels may help in the characterization and management of early breast cancer patients (Grassilli, 2014).

3.2 Vav1 down-modulates the Akt pathway

Abnormal activation of Akt pathways is the most common aberrations of signal transduction in solid tumors, including breast cancer (Mundi, 2016; Yang, 2016) in which the three known Akt isoforms (Akt1, Akt2, and Akt3) show a phenotype related expression (Clark and Toker, 2014; Iacovides, 2013). In particular, Akt1 has been demonstrated to suppress while Akt2 promotes breast cancer cell migration and invasion *in vitro* (Chin, 2010; Irie, 2005; Liu, 2006). The opposing function of Akt1 and Akt2 in cell migration and invasion was also demonstrated *in vivo* in mouse models. Similarly, activation of Akt1 decreases tumor metastatic dissemination but promotes mammary tumorigenesis in mouse models, whereas Akt2 primarily increases tumor metastasis in those models (Hutchinson, 2004; Dillon, 2009; Endersby, 2011). Moreover, specific Akt isoforms have been demonstrated to be drivers in particular cancers. In particular, Akt2, but not Akt1, mediates survival and maintenance of PTEN-deficient prostate cancer (Chin, 2014). Furthermore, activating mutations in Akt1 are much more common than those in Akt2 or Akt3, such as

the E17K mutation in the PH domain, being more than 25 fold less frequent in Akt2 or Akt3 than in Akt1 (Wang, 2018).

Epidemiological and preclinical studies confirmed that activation of Akt is implicated in the pathogenesis of breast cancer also by conferring resistance to systemic treatments (Yang, 2016) and a number of molecules have been generated to selectively or non-selectively inhibit the three isoforms (Mundi, 2016; Dey, 2017). Unfortunately, Akt inhibitors efficacy is particularly problematic in triple-negative breast cancers (TNBC), as they express all three Akt isoforms and show the highest activation of the Akt downstream pathways (Chin, 2014; Grottke, 2016; Massihnia, 2016).

On the basis of these evidences and of the above mentioned relationship between Vav1 and mRNA for Akt (Grassilli, 2014) the possible role of Vav1 in modulating the Akt pathway was investigated. Using both *in vitro* and *in vivo* models, we found that Vav1 down-modulates Akt acting at expression and/or activation levels depending on tumor subtype. The decreased p-Akt1 (Ser473) levels, indicative of its activation status, are a common effect of Vav1 up-modulation, suggesting that, in breast tumor derived cells and independently of their phenotype, Vav1 interferes with signaling pathways ended to specifically recruit Akt1. Only in ER-negative cell lines, the silencing of Vav1 induced the expression but not the activation of Akt2 (Grassilli, 2018).

A retrospective analysis of early invasive breast tumors allowed establishing the prognostic significance of the p-Akt/Vav1 relationship. In particular, we revealed that low Vav1 levels worsen the follow-up of patients with low p-Akt in their primary tumors and subjected to adjuvant chemotherapy (Grassilli, 2018). As the use of specific or pan Akt inhibitors may not be sufficient or may even be detrimental, increasing the levels of Vav1 could be a new approach to improve breast cancer outcomes. This might be particularly relevant for tumors with a triple-negative phenotype, for which target-based therapies are not currently available.

4. The tumor suppressor miR-29b

MicroRNAs (miRNAs) are endogenous, small, non-coding RNAs approximately 22 nucleotides in length with the ability to interfere in the expression of protein-coding messenger RNAs (mRNAs) by binding to the 3′-untranslated region (3′-UTR) of target mRNAs (Bartel, 2009; Auyeung, 2013). Because of their short sequence, these RNA molecules are able to mediate mRNA degradation by perfect base-pairing with target

mRNAs or by translational blockade via imperfect base-pair binding to a wide range of target genes. One miRNA might bind to a number of mRNA transcripts and, in turn, one mRNA can be targeted by a widespread panel of miRNAs. Consequently, miRNAs can dictate cell fate, cell cycle regulation, as well as cell proliferation, differentiation, apoptosis, and invasion (Pillai, 2007).

Accumulating evidence has demonstrated that miRNAs act as crucial regulators of cancer by targeting different signaling pathways and multiple mRNA transcripts. Moreover, a strong association of miRNA expression profiles with the etiology, classification, progression, and prognosis of multiple human cancers has been established and miRNAs can act as either oncogenes or tumor suppressors (Slack, 2008; Filipowicz, 2008).

The miR-29 family consists of miR-29a, miR-29b, and miR-29c, differing only in two or three bases. The miR-29 family is encoded and transcribed in tandem by two genes located on chromosome 7q32.3 (miR-29a/b1 locus) or chromosome1q32.2 (miR-29a/b2 locus), respectively. Mature miR-29b is therefore encoded by two distinct precursor stem sequences (pre-miRNA) on both chromosomes, a pre-miR-29b1 and pre-miR-29b2 stem. Although the sequences of the two pre-miR-29b stems are different, the mature miR-29bs, miR-29b1 and miR-29b2, resulting from these two stem structures are identical (Kole, 2011).

Overlapping functions of miR-29 family members include transcriptional regulation, epigenetic modulation and cell apoptosis. However, despite the high similarity of mature miR-29a/b/c sequences, numerous studies have showed that different isoforms of the miR-29 family may exert distinct functions (Han, 2010; Nguyen, 2011). Recent evidence suggested that aberrant expression of the members of the miR-29 family is involved in multiple cancers. (Yan, 2015).

Some recent studies assert that miR-29 family affects cell proliferation and apoptosis is dependent on the Akt pathway and p-Rb levels (Gong J, 2014). In multiple myeloma, the PI3K/AKT pathway is a negative regulator of miR-29b. Conversely, miR-29b behaves as a negative regulator of PI3K/AKT pathway by reducing Akt phosphorylation (Amodio, 2012; Yan, 2015). Furthermore, there is also evidence of miR-29b's role in cellular metabolism, including the regulation of both amino acid synthesis and insulin release (Mersey, 2005; Pullen, 2011). Teng and colleagues during them studies on ovarian cancer, using bioinformatics prediction, identified Akt2 and Akt3, but not Akt1, as potential downstream target genes of miR-29b, indicating that miR-29b-mediated effects on the Akt

signaling pathway and it is probably involved in cancer glycolysis and the Warburg effect (Teng, 2015).

4.1 MiR-29b in myeloid leukemia

MiR-29b is considered a tumor suppressor which plays a role in targeting leukemic oncogenes such as DNA methyltransferase 3A/B (DNMT3A/DNMT3B) in AML cells (Garzon, 2009). mMR-29b is generally down-regulated in AML and its restoration into AML cell lines or primary samples led to a dramatic reduction of tumorigenicity (Xu, 2014; Gong JN, 2014; Amodio, 2015). High pre-treatments levels of miR-29b have been recently associated with longer survival in AML patients treated with conventional chemotherapy and to improved clinical response to DNMT inhibitors, (Liu, 2010; Blum, 2010) suggesting that strategies aimed to increase this miRNA may be useful in therapeutic DNA hypomethylation of leukemic blasts.

As down-modulation of miR-29b correlates with different pathologies, a number of delivery systems for exogenous miR-29b have been generated (Yan, 2015; Pan, 2016; Pereira, 2017; Monaghan, 2017). Concerning AML, a transferrin-conjugated nanoparticle method was developed to increase miR-29b in AML blasts (Huang, 2013). However, despite its efficacy in *in vivo* AML models, this approach is not clinically available at present and activation of transcriptional expression may constitute an efficient therapeutic strategy for restoring the miR-29b level in myeloid leukemia. Different binding sites for several transcriptional factors have been identified in the promoter of miR-29a/b1 and miR-29b2/c clusters in different tissues (Amodio, 2015). In myeloid leukemia cells, CEBPα was reported to only regulate the miR-29a/b1 cluster, providing the rationale for miR-29b suppression in AML patients with loss of chromosome 7q or deficiency of this transcription factor (Eyholzer, 2010). Batliner et al. demonstrated that c-Myc directly represses miR-29b expression in APL cells, showing significantly lower miR-29b levels as compared to normal neutrophils. Accordingly, degradation of c-Myc is required for miR-29b upregulation that plays a crucial role during ATRA-induced differentiation of tumoral promyelocytes. Finally it has been demonstrated that both PU.1 and c-Myc are transcriptional regulators of the miR-29b2/c locus in APL cells and that low expression of miR-29b in APL is due to aberrant expression of these transcription factors (Batliner. 2012).

4.2 MiR-29b in breast cancer

In recent years, several studies have shown dysregulation of miR-29b in many types of tumors (Mulrane, 2013), such as gastric (Gong J, 2014), breast (Wang, 2011) and prostate (Takayama, 2015) cancer. As a member of the miR-29 family, miR-29b is generally recognized as a fundamental regulator of EMT, an event involved in cancer metastasis and chemoresistance (Sandhu, 2012). In solid tumors, miR-29b modulates many target genes, such as the DNMT family (Sandhu, 2014), oncogenes (Wang, 2015; Park, 2009) and tumor suppressor genes (Langsch, 2016; Zhu, 2016; Wang, 2017).

In breast cancer, low miR-29b expression is associated with larger tumor sizes and more advanced cancer stage (Shinden, 2015). As miR-29b was expressed in adjacent normal tissues and normal breast epithelial cell MCF-10A, it significantly lower expression in breast cancer cell lines, including MDA-MB-231 and MCF7, further proved that lower expression of miR-29b could be an indicator of breast cancer (Wang, 2017). Low expression of miR-29b has been found to have a significant association with poor disease-free survival (DFS) and poor overall survival (OS) in ER-positive and ER-negative breast cancer. This is consistent with previous *in vitro* and *in vivo* findings that miR-29b acts as a tumor suppressor miRNA (Park, 2009; Chou, 2013; Yang, 2013). In particular, Chou et al (Chou, 2013) showed that miR-29b is induced by GATA3 and inhibits metastasis by targeting genes involved in modifying the tumor microenvironment (ANGPTL4, LOX, MMP and VEGFA). Additionally, in ER-positive tumors, a significant inverse correlation between the expression levels of miR-29b and DNMT3A was identified (Shinden, 2015).

Aim

The general aim of the work described in the thesis is to assess the role of the multidomain protein Vav1 in up-modulating miR-29b that plays an onco-suppressor role in both myeloid leukemia and solid tumors also by inducing DNA hypomethylation.

On the basis of our previous data demonstrating that a Vav1/PU.1/miR-142-3p network sustains the ATRA-induced granulocytic differentiation of cells derived from acute promyelocytic leukemia (APL), the first part of the investigation was aimed to assess the role of PU.1 and Vav1 in regulating the expression of miR-29b in AML-derived cells, in order to identify new strategies that, up-modulating the miRNA, could be beneficial in DNA hypomethylation-based therapies. Our experiments were mainly performed with Kasumi-1 cells, showing the t(8;21) chromosomal translocation, that represents the most common cytogenetic subtype of AML. In this cell line, the ectopic expression of PU.1 overcomes its functional block induced by AML1-ETO, in turn involved in a regulatory circuit with miR-29b that controls the leukemic phenotype.

Once established that Vav1 is involved in up-modulation of miR-29b in AML-derived cells, our interest was focused on the study of the Vav1/miR-29b relationship in invasive breast tumors, in which both molecules have a positive prognostic value. As experimental model we used MDA-MB-231-derived cells stably expressing low or high levels of Vav1. On the basis of our previous studies demonstrating that Vav1 regulates the expression of specific Akt isoforms, in turn potential downstream targets of miR-29b, the presence of a Vav1/miR-29b/Akt axis was investigated in this cell model, to assess if up-regulation of Vav1 would be beneficial in treatment of breast tumors subtypes for which target-based therapies are not currently available.

Materials and Methods

All reagents were from Sigma (St Louis, MO, USA) unless otherwise indicated.

1. Cell culture and treatments

The human myeloid leukemia-derived Kasumi-1 (t(8;21)) and the APL-derived NB4 (t(15;17) cell lines (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France) and 1% penicillin-streptomycin solution (Gibco Laboratories). MDA-MB-231 cells were from the American Type Culture Collection (Rockville, MD, USA) and were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco Laboratories) supplemented with 10% FBS (Gibco Laboratories) and 1% penicillin-streptomycin solution (Gibco Laboratories).

MDA-MB-231 cells stably expressing Vav1 were obtained by transfection with a construct expressing the human full-length Vav1 and, after 48 h from transfection, cells were cultured for 3 weeks adding 1mg/ml G418 to the growth medium. The resistant cells were maintained in culture with a medium containing 0.1 mg/ml G418 and then used for cellular assays (Grassilli, 2014).

MDA-MB-231 cells stably silencing Vav1 (shVav1, clone 2271), as well as their control cells (clone shC002) were kindly provided by Dr. Tschan (Institute of Pathology, University of Bern).

All cells were grown at 37°C in a humidified atmosphere containing 5% CO2 in air. Sub-confluent cells were maintained at a density between $5x10^5$/mL and $1.5x10^6$/mL for Kasumi-1 and NB4 cells and between $2\times10^5/cm^2$ and $3\times10^5/cm^2$ for MDA-MB-231 cells and cell morphology was evaluated using an inverted phase-contrast microscope (Nikon, Melville, NY). Cells were monthly tested for mycoplasm and other contaminations and quarterly subjected to cell identification by single nucleotide polymorphism (SNP) typing.

Kasumi-1 and NB4 cells were treated for 4 days with 1 μmol/L ATRA dissolved in ethanol and Kasumi-1 cells were treated with 100 nmol/L phorbol 12-myristate 13-acetate (PMA) suspended in DMSO for 72 h .

To establish the percentage of adherent cells, after removal of cells in suspension, the cells adhering to the flask were detached with a trypsin/EDTA solution (Gibco Laboratories). Both suspended and adherent cells were counted using a hemocytometer in the presence of

trypan blue, in order to determine the number of viable cells and the level of adhesion was expressed as a percentage of adherent cells over the total number of cells.

2. Purification of nuclei

To obtain purified nuclei, NB4 and Kasumi-1 cells, after washing in PBS with 1 mmol/L Na_3VO_4, were resuspended in 10 mM Tris-HCl pH 7.4, 2 mM MgCl2, 10 mM NaCl (TM2) with protease inhibitors and incubated for 20 minutes in ice. After this time, 0.5% Triton X-100 was added and after 5 minutes the cellular solution was passed once through a syringe provided with a 22-gouge needle for NB4 or with a 20-gauge needle for Kasumi-1, causing an osmotic and mechanical shock necessary to obtain nuclei without nuclear membranes. The nuclei were subsequently stabilized by the addition of 3 mM $MgCl_2$ and subjected to centrifugation at 950-1000 rpm for 10 minutes. The nuclear pellet was then washed twice with a solution containing 10 mM Tris-HCl pH 7.4, 5 mM MgCl2, 10 mM NaCl (TM5) and protease inhibitors, to remove the detergent used for nuclear extraction and any residual cell debris (Nika, 2014).

3. Immunoprecipitation and Western blot analysis

For immunoprecipitation experiments, nuclei obtained from ATRA-treated NB4 and PMA-treated Kasumi-1 were lysed for 1 hour at 4°C in a buffer containing PBS 1X, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% Sodium Dodecyl Sulfate with protease inhibitors, incubated over night at 4°C with antibodies directed against Vav1 or PU.1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), immunoprecipitated with protein A-Sepharose (Pharmacia, Uppsala, Sweden) and finally resuspended in Laemmli's SDS sample buffer.
For Western blot analysis, total cell lysates were obtained by adding Laemmli's SDS sample buffer to cells after washing with cold PBS containing 1 mmol/L Na_3VO_4. Total cell lysates and immunoprecipitates from nuclei were separated on 8.5% polyacrylamide denaturing gels and blotted to nitrocellulose membranes (GE Healthcare Life Science, Little Chalfont, UK). The membranes were then reacted with antibodies directed against PU.1, Vav1, CEBP-α, Akt2 and GAPDH (Santa Cruz Biotechnology) and against β-tubulin (Sigma).
In particular, for the immuno-detection of PU.1, Vav1, CEBPα, Akt2 and GAPDH the membranes were incubated for 1 hour at room temperature with a solution of TBS containing 0.05% Tween-20 and 5% milk, in order to saturate the aspecific binding sites,

and subsequently were reacted with the primary antibody specific for the individual proteins diluted 1:1000. The membranes then, after appropriate washes with TBS added of 0.05% Tween-20, were incubated with a peroxidase-conjugated secondary antibody diluted 1:2000 in a TBS solution containing 0.05% Tween-20 and 5% milk for 45 minutes at room temperature.

For detection of β-Tubulin, membranes were saturated with a PBS buffer supplemented with 3% BSA for 1 hour at room temperature, followed by a reaction with the primary antibody diluted 1:1000 in PBS containing 0.1% Tween-20 and 0.1% BSA for 2 hours. After 3 washes with PBS added with 0.1% Tween-20, to remove unbound antibody, the hybridization was carried out with the secondary antibody conjugated with peroxidase diluted 1:2000 in a PBS solution containing 0.1% Tween-20 and 0.1% BSA for 45 minutes at room temperature.

The immunocomplexes were detected by chemiluminescence using the ECL system (Perkin-Elmer, Boston, MA), according to the manufacturer's instructions.

The chemiluminescence-derived bands were acquired with ImageQuantTM LAS 4000 biomolecular imager (GE Healthcare Life Science), and the densitometrical analysis was performed by means of Image Quant TL software (GE Healthcare Life Science).

To make nitrocellulose membranes available for subsequent reactions with different antibodies it was necessary to remove the primary antibody/secondary antibody complex by means of incubation at 50 °C for 10 minutes with a stripping solution consisting of 100 mM β-mercaptoethanol, 62.5 mM Tris-HCl pH 6.8 and 2% SDS, followed by washing in TBS containing 0.1% Tween-20 for 10 min.

4. Down-modulation of Vav1 and PU.1 expression

The down-modulation of the expression of Vav1 and PU.1 proteins in Kasumi-1 and NB4 cells was performed by using the RNA Interference technique, following a previously reported procedure (Grassilli, 2016). In particular, a pool of 3 target-specific 20-25nt siRNAs specific for Vav1 and PU.1 mRNAs, respectively, was designed by Santa Cruz Biotechnology (Santa Cruz Biotechnology) The lyophilized siRNAs mixtures were dissolved in sterile suspension buffer to obtain a 10 μM stock solution.

After 24 hours from seeding, the cells were transfected with siRNAs by electroporation according to the following procedure: 80 pmol of siRNAs were added to 2 x 10^6 cells in 100 μl of RPMI 1640 containing 20% FBS in cuvettes for electroporation (4MM Cuvette

W/IN, Gene Pulser, Bio-Rad Laboratories, Hercules, CA). The cells were then electroporated (250 V for 25 msec for Kasumi-1 and 250 V for 30 msec for NB4) with the Electro Square Porator ECM 830 electroporator (BTX, Genetronics, Inc, San Diego, CA), then diluted in 600 µl of RPMI 1640 containing 20% of FBS. As a control for transfection efficiency, which was always higher than 60%, a non-silencing fluorescein-labelled duplex RNA (Qiagen S.p.A, Milan, Italy) was used. 5 hours after transfection, cells were treated with ATRA or PMA, incubated at 37°C in a 5% CO_2 atmosphere and then subjected to immunochemical or immunocytochemical analysis, to miR-29b evaluation and to quantitative chromatin immunoprecipitation experiments.

5. Modulation of miR-29b expression

The modulation of miR-29b expression was performed by transient transfection of MDA-MB-231 cells with a synthetic inhibitor or a mimic specific for this miRNA (miRVana miRNA, Life Technologies, Monza, I).

Transfection was performed using 1 mg/ml Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Briefly, the day before transfection, cells were seeded at 2×105 cells/cm2 into six-well plates and when cells reached 40-45% confluence were mixed with 60nM inhibitor or 30nM mimic and 5 µl of Lipofectamine 2000 in 100 µl of Opti-MEM I medium (Gibco Laboratories). Random sequences (Life Technologies) were used as negative controls and to check for any contribution from miRNA in serum.

After 5 hours of incubation at 37°C in a 5% CO_2 atmosphere, transfected cells were cultured in growing medium for 48 h prior to immunochemical and cellular assays.

6. Quantitative Real-time PCR (qRT-PCR) assay

High-quality small RNA from cells under different experimental conditions was extracted using a miRNeasy Micro Kit (Qiagen), according to the manufacturer′s instruction.

To analyze miRNAs expression levels, 10 ng of RNA was subjected to single-stranded cDNA synthesis and the obtained cDNAs were employed as templates for quantitative Real-time PCR-based miR-29b expression measurements using TaqMan MicroRNA Assays (ID 000413; Life Technologies). Thermal cycling and fluorescence detection were performed, according to the manufacturer’s instructions, using a Bio-Rad CFX96TM sequence detection system (Bio-Rad Laboratories), and the data were analysed using a

dedicated software (Bio-Rad Laboratories). miRNA expression levels were normalized to U6 snRNA (Life Technologies), and fold change was determined using the $2^{-\Delta\Delta Ct}$ method

To evaluate expression of pri-miR-29b1/2, pre-miR-29b1/2 and mRNA for Vav1, Akt2 and Akt3, 1000 ng of total RNA were reverted to cDNA using the "High Capacity cDNA Reverse Transcription" kit (Applied Biosystems) according to the manufacturer's instructions.

For qRT-PCR the following primers were used:

i) Vav1 (Fw: 5'-ACGTCGAGGTCAAGCACATT-3' Rev: 5'-GGCCTGCTGATGGTTCTCTT-3'),

ii) pri-miR-29b1 (Fw:5'-ATGGCAGTCAGGTCTCTG-3' Rev: 5'-GCAATGCAAATGTATGCAAAT-3'),

iii) pri-miR-29b2 (Fw: 5'- TTGAGTGTGGCGATTGTCAT-3' Rev: 5'-ATCAACGCCGAATACTCCAG-3'),

iv) pre-miR-29b1 (Fw: 5'- TGCCAAAGCTCTGTTTAGACCA-3' Rev: 5'-GGTCTCCCCCAAGAACACTG-3'),

v) pre-miR-29b2 (Fw: GAGGCTGGGTCTTCCGATTG-3' Rev: 5'-AGGAGAGGAGCCAGTTCCAT-3')

vi) GAPDH (Fw: 5'-GAAGGTGAAGGTCGGAGTC-3' Rev: 5'-GAAGATGGTGATGGGATTTC-3')

vii) RPL-32 (Fw: 5'- CATCTCCTTCTCGGCATCA-3' Rev: 5'-AACCCTGTTGTCAATGCCTC-3').

After adding to each tube 5 µl of iTaq Universal SYBR green SuperMix (Bio-Rad Laboratories), the thermal cycling and fluorescence detection were performed by programming the following steps: 2 minutes at 50 °C, 2 minutes at 95 °C and, repeated for 40 cycles, 15 seconds at 95 °C and 1minute at 60 °C (Melt Curve 65 °C – 95 °C increment 0.5 °C for 5 seconds). Bio-Rad CFX96^{TM} sequence detection system (Bio-Rad Laboratories) was used and the data were analysed using a dedicated software (Bio-Rad Laboratories).

For evaluation of Akt2 (ID hs00609846; Life Technologies) and Akt3 (ID hs00987343; Life Technologies) mRNA levels by qRT-PCR, the appropriate TaqMan probe was purchased and the values were normalized to RPL-13A (ID hs03043885; Life Technologies) gene. Thermal cycling and fluorescence detection were performed,

according to the manufacturer's instructions, using a Bio-Rad CFX96TM sequence detection system and the data were analysed using a dedicated software (Bio-Rad Laboratories).

Vav1 mRNA expression levels were normalized to the expression of GAPDH, pri- and pre-miR-29b1/2 levels were normalized to RPL-32 mRNA levels and fold changes were determined using the $2^{-\Delta Ct}$ method. Cycle threshold >35 was excluded. Control PCR samples were run without cDNA. All reactions were performed in triplicate, and the experiments were repeated 3 times.

7. Quantitative chromatin immunoprecipitation (Q-ChIP) assay

Quantitative chromatin immunoprecipitation experiments were performed with untreated and treated Kasumi-1 and NB4 cells and with MDA-MB-231 in control conditions and in which the expression of Vav1 was modulated, using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY, USA), as previously reported (Brugnoli, 2009; Grassilli, 2016).

Briefly, the samples were fixed with 1 % formaldehyde to cross-link the DNA with proteins, lysed and sonicated to obtain DNA fragments of 200–1000 bp. After pre-clearing with salmon sperm DNA/protein-A agarose beads, were subjected to immunoprecipitation at 4°C overnight with antibodies directed against PU.1, Vav1 or CEBPα (Santa Cruz Biotechnology) or with a non-specific IgG (Santa Cruz Biotechnology), used as a negative control. Beads were then washed, protein/DNA complexes eluted and cross-links reversed by heating samples at 65°C overnight. After protein digestion, DNA was recovered using a PCR purification kit (Promega, Madison, WI, USA) in 50 µL elution buffer.

Quantitative PCR of (i) a 170-bp DNA fragment, encompassing the putative PU.1 binding site located at -330/-324 bp from the transcriptional start in the human pri-miR-29a/b1 promoter on chromosome 7q32.3, of (ii) a 181-bp DNA fragment, encompassing the putative PU.1 binding site located in the proximal miR-29b2/c promoter on chromosome 1q32.2 and of (iii) a 131-bp DNA fragment, encompassing the putative CEBPα binding site located at -89/+42 bp from the transcriptional start in the human pri-miR29a/b1 promoter on chromosome 7q32.3, was performed in triplicate using an iTaq Universal SYBR green SuperMix on a Bio-Rad CFX96TM Real-time detection system (Bio-Rad Laboratories).

The primers used were as follows:

(i) Fw: 5'-GCAGAGGATTAGACAGAGGGTG-3', Rev: 5'-CTGAGAAGTGAGCAGCAACC-3'; (ii) Fw: 5'-GTTCTTCCCTGGACTTCTCG-3' , Rev: 5'-AAGCTGGTTTCACATGGTGG-3'; (iii) Fw: 5'-GCAGGTTTTCAGTTGGTGGTTT-3', Rev: 5'-GCCGTGACAGTTCAGTAGGA-3'. Input corresponding to 1% of the total sonicated DNA was used as a positive control. All experiments were performed in triplicate.

To verify the ChIP specificity, samples from PMA-treated Kasumi-1 cells were subjected to PCR using primers (iii) amplifying a region flanking the PU.1 binding site in the human pri-miR-29a/b1 promoter on chromosome 7, which was not predicted to bind PU.1. ChIP-qPCR data are presented as relative to input signals and in comparison with the background signals (IgG). PCR products were separated on tris-acetate 1% agarose gels, stained with ethidium bromide and visualized by UV light apparatus.

8. Immunocytochemical analysis

ATRA-treated NB4 cells were placed onto round 12-mm glass coverslips by means of cytocentrifugation (Cytospin 3; Shandon Scientific, Astmoor, UK). Untreated and ATRA-treated Kasumi-1 cells were placed on glass dishes coated with 100 µg/mL Poly-L-Lysine to promote attachment of cells. PMA-treated Kasumi-1 cells that acquired adhesion capability were allowed to grow directly on coverslips.

Samples on glass dishes were fixed with freshly prepared 4% paraformaldehyde (10 minutes at room temperature) and washed in PBS. For the analysis of the distribution of Vav1 and PU.1, the cells fixed on the slide were first incubated with a NET Gel solution (150 mM NaCl, 5 mM EDTA, 50 mM TRIS-HCl pH 7.4, 0.05% NP40, 0.25% Carrageenan Lambda gelatine and 0.02% Na azide) for 1 hour at room temperature in order to block non-specific binding sites and allow penetration of the antibody inside the cell. Subsequently the cells were incubated with the antibodies directed specifically against proteins of interest diluted 1:20 in NET Gel for 3 hours at room temperature. After 3 washes with NET Gel, the samples were incubated with FITC- and/or TRITC-conjugated secondary antibodies for 45 minutes at room temperature in the dark. After one wash with NET gel and one wash with PBS, samples were reacted for 1 minute with 0.5 mg/mL 40,6-diamidino-2-phenylindole (DAPI), dried with a progressive serie of ethanol (70%, 90% and 100%) and mounted in glycerol containing 1,4- diazabicyclo [2.2.2] octane (DABCO)

to retard fading. Fluorescent samples were observed with a Nikon Eclipse TE2000-E microscope (Nikon, Florence, Italy), acquiring cell images by the ACT-1 software for a DXM1200F digital camera (Nikon) (Grassilli, 2014).

For confocal analysis, after the labelling with secondary antibodies, samples were washed and incubated with TO-PRO®-3 Stain (Thermo Fisher Scientific, Paisley, UK), dried with ethanol and mounted in glycerol containing DABCO. Images were obtained using an Axiovert 220M confocal microscope equipped with a 1009 oil immersion Plan-Neofluar objective (NA 1.3, Carl Zeiss, Göttingen, Germany) and a CoolSnap HQ CCD camera (Carl Zeiss).

9. Statistical analysis

Statistical analysis for mRNA, miRNA and protein expression levels was performed using the 2-tailed Student's t test for unpaired data with the GraphPad Prism 6.0 statistical package (GraphPad Software, San Diego, CA, USA). P values < 0.05 were considered statistically significant.

For correlation plot we calculated Pearson's correlation coefficient (r) and p value and the Spearman's rank correlation coefficient (ρ) and p value, for all expression values of interest genes and miRNAs. The analysis was performed comparing one gene or miRNA to all others within each sample of cancer dataset (TGCA). In this study, we consider significant ρ with p value <0.05. JASP was the software used for correlation processing obtained by LOG2 RPM.

Results

1. Vav1 is necessary for PU.1 mediated up-modulation of miR-29b in acute myeloid leukemia-derived cells

The aim of the first part of the data reported in the thesis was to assess the involvement of Vav1 in the upregulation of miR-29b by means of the transcription factor PU.1 in non-APL cells.

1.1 PMA, but not ATRA, induces a PU.1 dependent increase of miR-29b levels in Kasumi-1 cells

In order to verify the ability of PU.1 to modulate the expression of miR-29b in non-APL myeloid cells, we used as experimental model the Kasumi-1 cell line, representing the most common cytogenetic subtype of AML. Kasumi-1 cells show in fact the t(8;21) chromosomal translocation which encodes the AML1-ETO fusion protein.

On the basis of the described PU.1-mediated expression of miR-29b induced by ATRA in NB4 cells (Batliner, 2012), this agonist was firstly administered to Kasumi-1. As expected (Manfredini, 1999), ATRA induces a slight cell adhesion, indicative of a partial differentiation along the monocyte-macrophage lineage, that reaches 18% of the cells after 3 days of treatment (Figure 5A). Immunochemical analysis revealed a significant increase of PU.1 (Figure 6A, 6B). However, unlike what we observed in NB4 cells, ATRA was not able to increase the miR-29b level in Kasumi-1, at all the investigated times (Figure 5B, Figure 7).

By treating Kasumi-1 cells with PMA, known to increase (Batliner, 2012) or activate (Park, 2016) PU.1 in both APL and non-APL-derived cell lines, we found a significant increase of its expression (Figure 6A, 6B), in parallel with cells adhesion close to 90% (Figure 5C). Unlike ATRA treatment, PMA was able to induce a significant increase of miR-29b expression, that reached the maximum after 3 days of treatment (Figure 5D, Figure 7).

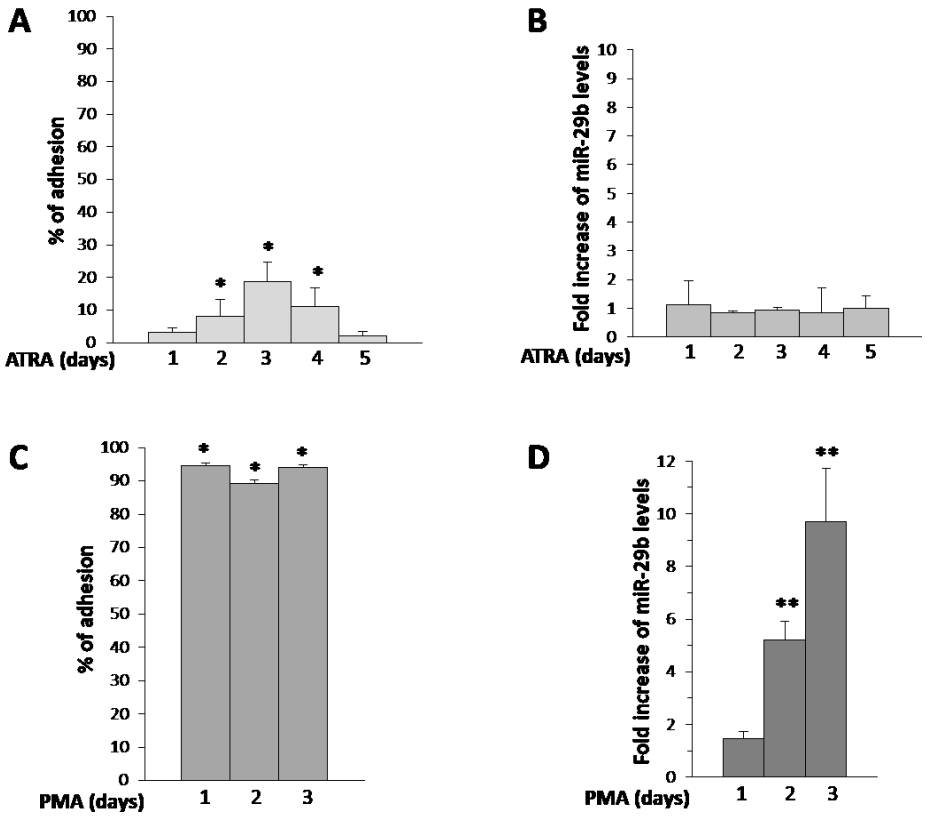

Figure 5. Adhesion and miR-29b levels in agonists-treated Kasumi-1 cells. Kasumi-1 cells treated with ATRA or PMA for the indicated times (days) were evaluated for their adhesion capability (A, C) expressed as percentage of adherent cells over the total number of cells, and miR-29b expression (B, D). MiR-29b levels are shown as fold changes relative to the untreated condition and represent the means of 3 separate experiments ± SD. *P <0.05, **P <0.01 compared to untreated.

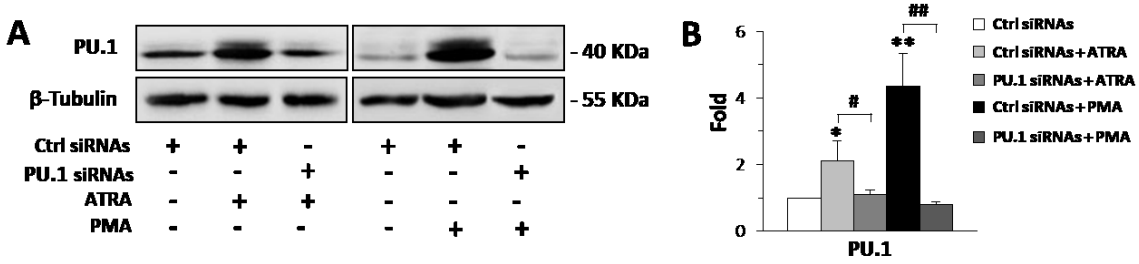

Figure 6. Modulation of PU.1 in agonist-treated Kasumi-1. (A) Representative Western blot analysis using the indicated antibodies of Kasumi-1 cells in which PU.1 was down-regulated during 72 hours of ATRA or PMA treatment. (B) Relative amounts of PU.1 as deduced from the densitometry of Western blot bands normalized with β-Tubulin. Ctrl siRNAs: scramble siRNAs; PU.1 siRNAs: siRNAs specific for PU.1. The mean expression level of three separate experiments ± SD is shown. *P <0.05, **P <0.01 compared to respective controls (Ctrl siRNAs). #P <0.05, ##P <0.01.

Since PU.1 regulates miR-29b in APL-derived cells (Batliner, 2012), experiments were conducted to establish if this event also occurs in non-APL-derived cells. For this purpose, Kasumi-1 cells were transiently transfected with siRNAs specific for PU.1 and treated with ATRA or PMA. As shown in Figure 6, the silencing of PU.1 was effective in counteracting the protein accumulation and significantly inhibited the increase of miR-29b expression induced by PMA (Figure 7).

On the other hand, the down-modulation of PU.1 also decreased the basal expression of miR-29b in Kasumi-1 cells, confirming its effective role in regulating the miRNA expression in this cell line (Figure 7).

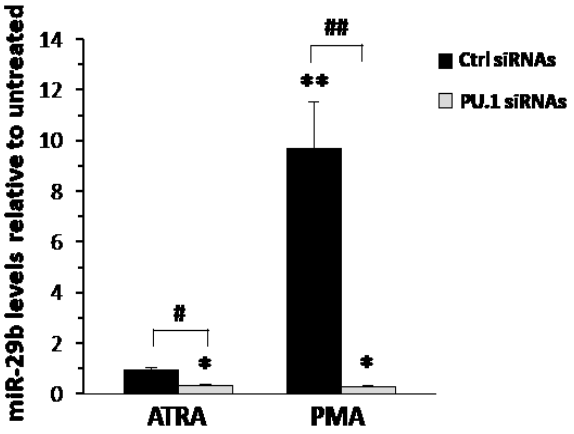

Figure 7. Agonist-induced modulation of miR-29b levels in Kasumi-1. qRT-PCR analysis of miR-29b levels in Kasumi-1 cells in which PU.1 was down-regulated during treatment with ATRA or PMA. The values re shown as fold changes relative to the untreated condition, and represent the means of 3 separate experiments ± SD. Ctrl siRNAs: scramble siRNAs; PU.1 siRNAs: siRNAs specific for PU.1*P <0.05, **P <0.01 compared to untreated conditions. #P <0.05, ##P <0.01.

This first bulk of data indicated that the transcription factor PU.1 is involved in the regulation of miR-29b also in non-APL myeloid leukemia derived cells.

Since the expression of miR-29b comes from the contribution of 2 different loci on chromosome 7q32.3 (miR-29b1) and on chromosome 1q32.2 (miR-29b2) (Yan, 2015), the *in vivo* binding of PU.1 to both promoters was investigated by Chromatin Immunoprecipitation (ChIP) assays performed with two pairs of primers that amplifies the potential PU.1 binding regions (Figure 8A). As shown in Figure 8B, PU.1 was selectively recruited to the miR-29b locus on Chromosome 7 also in control conditions and PMA, but not ATRA, led to a significant increase of DNA associated to the transcription factor.

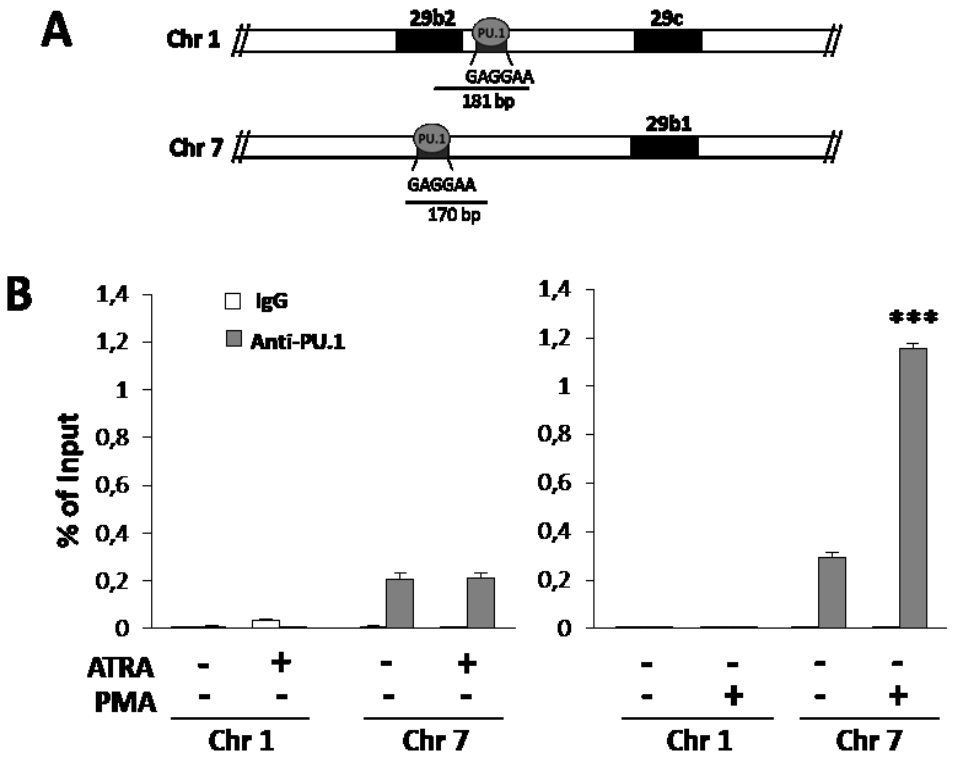

Figure 8. ***In vivo*** **interaction of PU.1 with miR-29b promoters in agonist treated Kasumi-1.** (A) Schematic representation of the putative PU.1 binding sites within the human miR-29c/b2 promoter on Chr 1q32.2 and within the human miR-29a/b1 promoter on Chr 7q32.3. (B) Analysis of *in vivo* recruitment of PU.1 to both miR-29b promoters performed by chromatin immunoprecipitation with an antibody directed against PU.1 in Kasumi-1 cells treated with ATRA or PMA. The data are shown as percentage of the Input (genomic DNA collected before immunoprecipitation). IgG: negative control. Values represent the means of 3 separate experiments ± SD. ***P <0.001 compared to untreated cells.

The specific binding of PU.1 was verified by performing a ChIP assay on a region of DNA flanking the miR-29b1 promoter on Chromosome 7, without predicted sequences able to interact with the transcription factor (Figure 9).

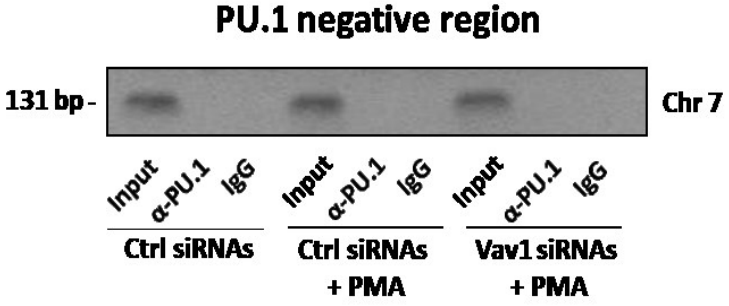

Figure 9. ChIP assay on a PU.1 negative region. Representative analysis of chromatin immunoprecipitation with an antibody directed against PU.1 in Kasumi-1 cells in which Vav1 was down-regulated during PMA treatment. The bands correspond to the PCR products obtained amplifying a region of 131 bp containing a sequence located in the miR29a/b1 promoter on Chr 7 not predicted to bind to PU.1. Ctrl siRNAs: scramble siRNAs; Vav1 siRNAs: siRNAs specific for Vav1. Input: genomic DNA not subjected to immunoprecipitation (positive control); IgG: samples immunoprecipitated with a non-specific IgG (negative control).

Therefore, we can conclude that, at least in this cell model of non-APL myeloid leukemia, PU.1 is directly involved in the transcription of miR-29b1 and that PMA is an agonist able to induce increased binding of the transcription factor to its consensus sequence on Chromosome 7, allowing to increase miRNA level.

1.2 In NB4 cells, Vav1 is essential for binding of PU.1 to its consensus sequences on the miR-29b promoters

As we previously found that, in APL-derived cells, Vav1 regulates the presence of PU.1 on its consensus region on the miR-142-3p promoter (Grassilli, 2016), our subsequent experiments were aimed to assess whether, also in regulation of miR-29b, the PU.1 action is supported by Vav1.

We firstly performed the study in NB4 cells treated with ATRA in which, as expected (Brugnoli, 2010; Batliner, 2012), we found an increase of both PU.1 and Vav1 (Figure 10), as well as of miR-29b (Figure 11).

The agonist-induced expression of the miRNA was almost completely inhibited by silencing PU.1 or Vav1 with specific siRNAs (Figure 10A, Figure 11), confirming the involvement of both proteins in miR-29b expression in this cell model.

ChIP assays with an anti-PU.1 antibody were then performed, which did not reveal DNA associated to the transcription factor in control conditions. At variance, ATRA treatment induced recruitment of PU.1 to miR-29b promoters (Figure 12). When cells were subjected to Vav1 down-modulation the interaction of PU.1 with DNA was abolished (Figure 12), indicating that, also in this context, the presence of Vav1 is necessary for the ATRA-mediated PU.1 recruitment to DNA.

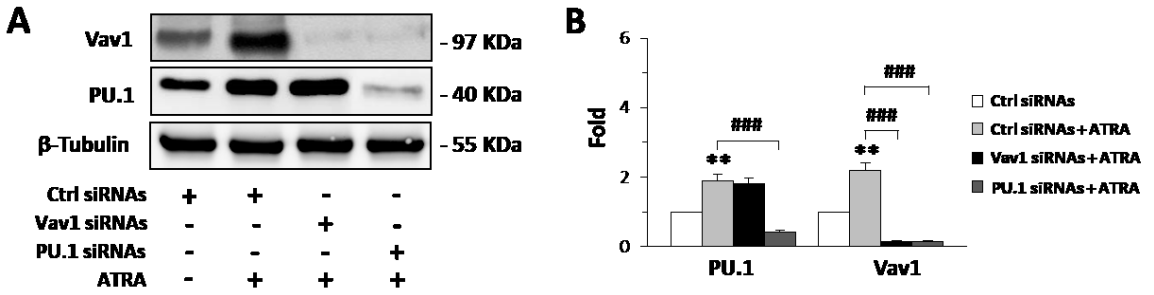

Figure 10. Vav1 and PU.1 expression in ATRA-treated NB4 cells. (A) Representative Western blot analysis, using the indicated antibodies, of NB4 cells in which PU.1 or Vav1 were down-modulated during 96 hours of ATRA treatment. (B) Relative amounts of PU.1 and Vav1, as deduced from the densitometry of Western blot bands normalized with β-Tubulin, used as internal control for equivalence of loaded proteins. The mean expression level of three separate experiments ± SD is shown. Ctrl siRNAs: scramble siRNAs; PU.1 siRNAs: siRNAs specific for PU.1; Vav1 siRNAs: siRNAs specific for Vav1 ± SD. **P <0.01 compared to respective controls (Ctrl siRNAs) taken as 1. ###P <0.001.

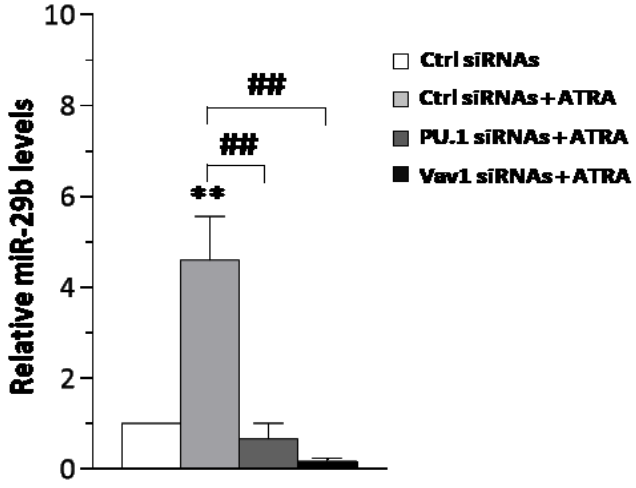

Figure 11. MiR-29b expression in ATRA-treated NB4 cells. (A) qRT-PCR analysis of miR-29b levels in NB4 cells in which PU.1 or Vav1 were down-regulated during 96 hours of ATRA treatment. The values are shown as fold changes relative to the untreated condition by using the $2^{-\Delta\Delta CT}$ method and represent the means of 3 separate experiments ± SD. Ctrl siRNAs: scramble siRNAs; PU.1 siRNAs: siRNAs specific for PU.1; Vav1 siRNAs: siRNAs specific for Vav1. **P <0.01 compared to respective controls (Ctrl siRNAs) taken as 1. ##P <0.01.

In order to verify the presence of Vav1 in molecular complexes on the consensus sequences for PU.1 in miR-29b promoters, ChIP experiments were performed using an anti-Vav1 antibody, which did not show significant amounts of DNA either in control and ATRA treated conditions (data not shown).

The subcellular relationship between PU.1 and Vav1 was subsequently analyzed by confocal microscopy in NB4 cells treated with ATRA and stained with anti-PU.1 and anti-Vav1 antibodies.

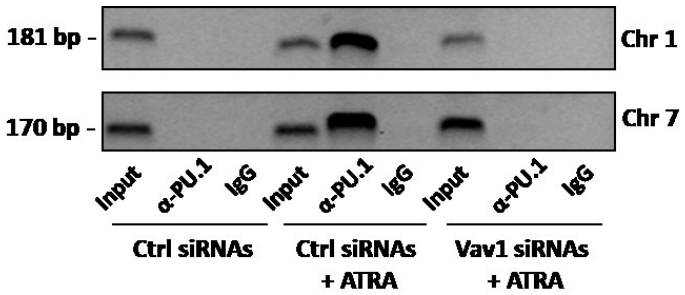

Figure 12. *In vivo* interaction of PU.1 with miR-29b promoters in ATRA treated NB4 cells. Representative analysis of *in vivo* recruitment of PU.1 to human miR-29c/b2 and miR-29a/b1 promoters by chromatin immunoprecipitation with an antibody directed against PU.1 in NB4 cells in which Vav1 was down-regulated during ATRA treatment. The bands correspond to PCR products obtained amplifying a 181 bp DNA fragment, encompassing the putative PU.1 binding site within the human miR-29c/b2 promoter on Chr 1 and a 170 bp DNA fragment encompassing the putative PU.1 binding site located in the proximal miR29a/b1 promoter on Chr 7. Ctrl siRNAs: scramble siRNAs; Vav1 siRNAs: siRNAs specific for Vav1. Input: genomic DNA not subjected to immunoprecipitation (positive control); IgG: samples immunoprecipitated with a non-specific antibody (negative control).

As shown in Figure 13A, PU.1 and Vav1 revealed an almost exclusive co-localization inside the nucleus, within large speckled agglomerations.

Co-immunoprecipitation experiments carried out with anti-PU.1 and anti-Vav1 antibodies confirmed our previous data (Brugnoli, 2010) demonstrating the nuclear association of the two molecules in ATRA treated NB4 (Figure 13B).

This bulk of data suggests that, even if Vav1 is not present in the molecular complex with PU.1 on the miR-29b promoters, the PU.1/Vav1 cooperation inside the nuclear compartment is necessary for the miR-29b expression induced by ATRA in APL-derived cells.

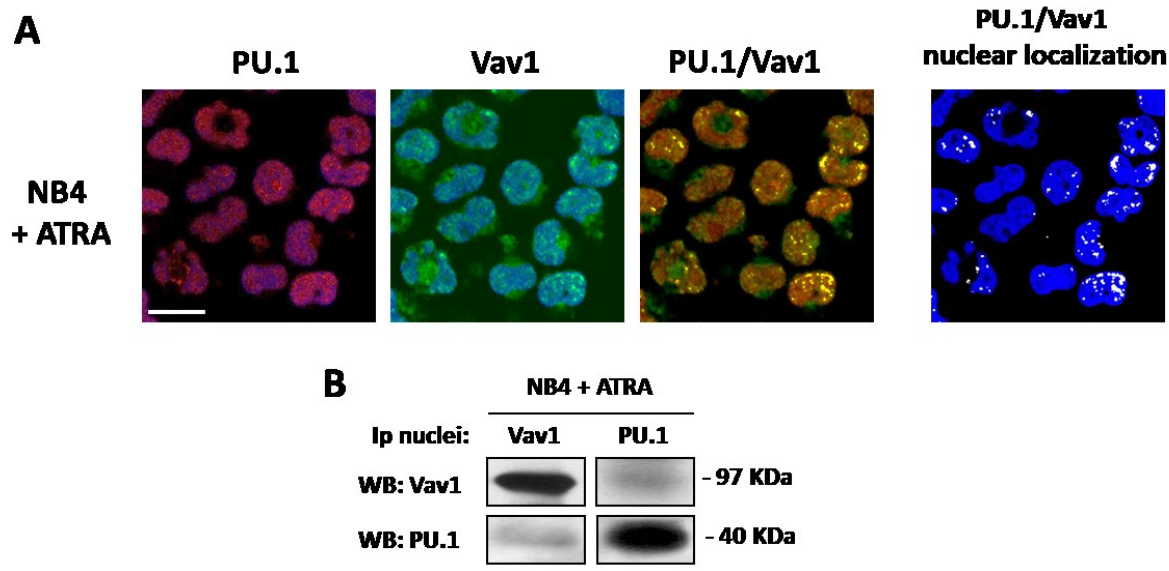

Figure 13. Vav1 and PU.1 association in ATRA-treated NB4 cells. (A) Representative confocal images of NB4 cells treated with ATRA and stained with antibodies against Vav1 (green staining) and PU.1 (red staining). TO-PRO®-3 Stain was used to counterstain the nucleus (shown in blue). PU.1 and Vav1 images are shown as the overlay of the protein staining (red or green) with the staining of the nucleus (blue). Merged PU.1/Vav1 staining is shown with co-localization resulting in yellow. To the right, PU.1/Vav1 co-localization points were white colored and overlapped to nuclear staining (blue). Bar = 20 µm.
(B) Representative Western blot analysis with the indicated antibodies of Vav1 and PU.1 immunoprecipitates from nuclei of NB4 cells treated with ATRA. The data are representative of three separate experiments.

1.3 In Kasumi-1 cells, miR-29b expression requires the presence of Vav1 in molecular complexes associated with the PU.1 binding site

Based on results obtained with NB4 cells and above reported, the involvement of Vav1 in miR-29b expression was investigated in Kasumi-1 cells treated with ATRA or PMA. We found that, although in this cell line Vav1 is only up-modulated by ATRA treatment, its silencing (Figure 14) reduced the basal expression of miR-29b level in cells treated with ATRA and completely abrogated the increase of the miRNA induced by PMA (Figure 15). This is indicative of the need of adequate amount of Vav1 for the miR-29b expression also in this cell line.

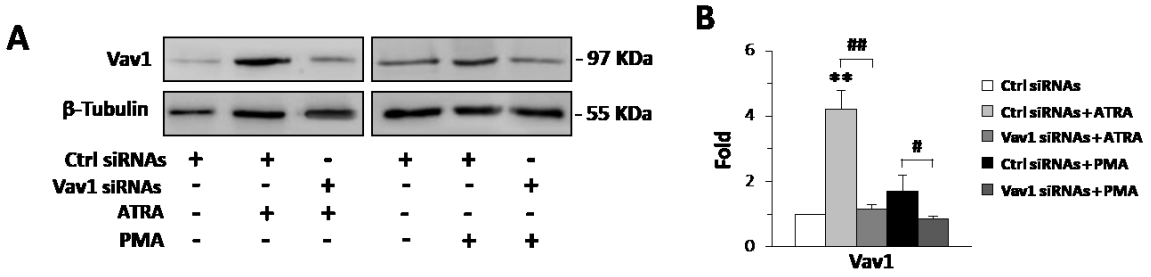

Figure 14. Down-modulation of Vav1 in agonist-treated Kasumi-1 cells. (A) Representative Western blot analysis with the indicated antibodies of Kasumi-1 cells in which Vav1 was down-regulated during 72 hours of ATRA or PMA treatment. (B) Relative amounts of Vav1 as deduced from the densitometry of Western blot bands normalized with β-Tubulin, used as internal control for equivalence of loaded proteins. The mean expression level of three separate experiments ± SD is shown. Ctrl: scramble siRNAs; Vav1 siRNAs: siRNAs specific for Vav1. **P <0.01 compared to Ctrl siRNAs. #P <0.05, ##P <0.01.

Unlike what we observed in NB4 cells treated with ATRA, the ChIP assay performed with the anti-PU.1 antibody showed that the interaction of the transcription factor with the miR-29b promoter on Chromosome 7 is not compromised in Kasumi-1 cells silenced for Vav1 during treatment with PMA (Figure 16A).

Interestingly, ChIP analysis performed with the anti-Vav1 antibody and using primers able to amplify the PU.1 recognizing region within the miR-29b promoters revealed significant amounts of DNA in Vav1 immunoprecipitates from both untreated and PMA treated Kasumi-1 cells (Figure 16B). This suggests that, unlike NB4 cells, the expression of miR-29b in Kasumi-1 requires the presence of Vav1 in the molecular complexes on the PU.1 binding site on Chromosome 7.

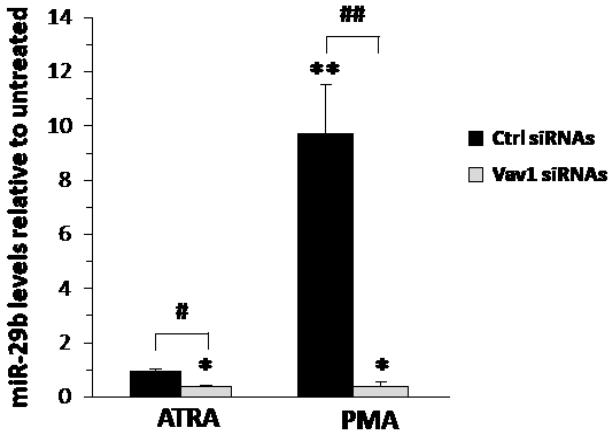

Figure 15. MiR-29b levels in Vav1-silenced Kasumi-1 cells. qRT-PCR analysis of miR-29b levels in Kasumi-1 cells in which Vav1 was down-regulated during ATRA or PMA treatment. The values are shown as fold changes relative to the untreated condition and represent the means of 3 separate experiments ± SD. Ctrl siRNAs: scramble siRNAs; Vav1 siRNAs: siRNAs specific for Vav1 *P <0.05, **P <0.01 compared to untreated. #P <0.05, ##P <0.01.

In order to assess if the intracellular localization of Vav1 could justify the lack of effects of PU.1 on miR-29b expression induced by ATRA in Kasumi-1 cells, the immunocytochemical analysis of untreated and agonist-treated cells was performed. As reported in Figure 17A the untreated population is heterogeneous and comprises cells expressing different levels of PU.1, which is mainly localized within the nucleus, according to its transcriptional role, and increased after both ATRA and PMA administration.

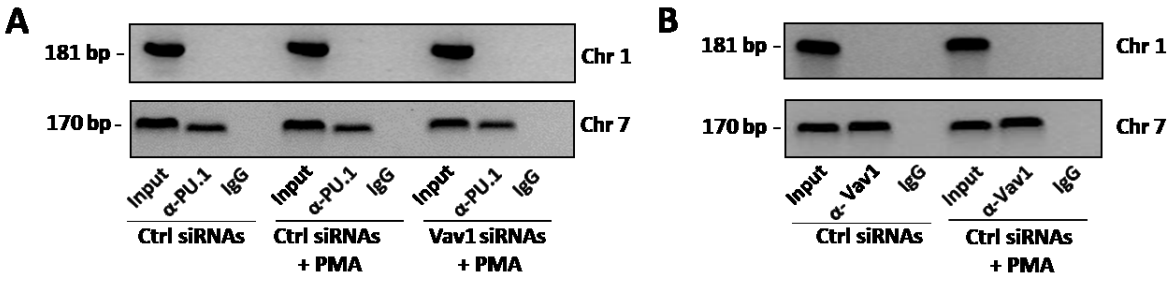

Figure 16. *In vivo* interaction of PU.1 and Vav1 with miR-29b promoters. (A) Representative analysis of *in vivo* recruitment of PU.1 to human miR-29c/b2 and miR-29a/b1 promoters by chromatin immunoprecipitation in Kasumi-1 cells in which Vav1 was down-regulated (Vav1 siRNAs) during PMA treatment. The bands correspond to PCR products obtained amplifying a 181 bp DNA fragment, encompassing the putative PU.1 binding site within the human miR-29c/b2 promoter on Chr 1 and 170 bp DNA fragment encompassing the putative PU.1 binding site located in the proximal miR29a/b1 promoter on Chr 7. (B) Representative analysis of in vivo recruitment of Vav1 to human miR-29c/b2 and miR29a/b1 promoters in the same cell model. Chromatin fragments were obtained by immunoprecipitation with an antibody directed against Vav1 and DNA was amplified by PCR. Ctrl siRNAs: scramble siRNAs. Input: genomic DNA not subjected to immunoprecipitation (positive control); IgG: samples immunoprecipitated with a non-specific IgG (negative control).

Concerning Vav1, a cytoplasmic localization of the protein, describing the nuclear periphery, was observed in both untreated and ATRA-treated cells, while PMA induced the accumulation of Vav1 inside the nuclear compartment, in which it showed a dotted staining (Figure 17A).

Confocal immunofluorescence analysis of Kasumi-1 cells treated with PMA and simultaneously reacted with anti-PU.1 and anti-Vav1 antibodies allowed to highlight a number of PU.1/Vav1 co-localization spots inside the nucleus (Figure 17B).

In conclusion, our data allowed to assess that the PU.1/Vav1 association inside the nucleus of PMA-treated Kasumi-1 justifies the presence of both molecules in the protein/DNA complexes on the miR-29b1 promoter and underlining their possible cooperation in different transcriptional activities. The lack of nuclear accumulation of Vav1 following ATRA treatment could therefore explain the ineffectiveness of this agonist on miR-29b expression in this cell model. Our data also suggest that, even if PU.1 is directly responsible of miR-29b regulation at transcriptional level, the PU.1/Vav1 association inside the nuclear compartment is a crucial event in the expression of a mature microRNA.

Altogether, the above reported data allowed us to assess that PU.1 can regulate the expression of miR-29b in both APL- and non-APL- derived cell lines and that this event is almost completely dependent on Vav1, that plays a cell-dependent contribution.

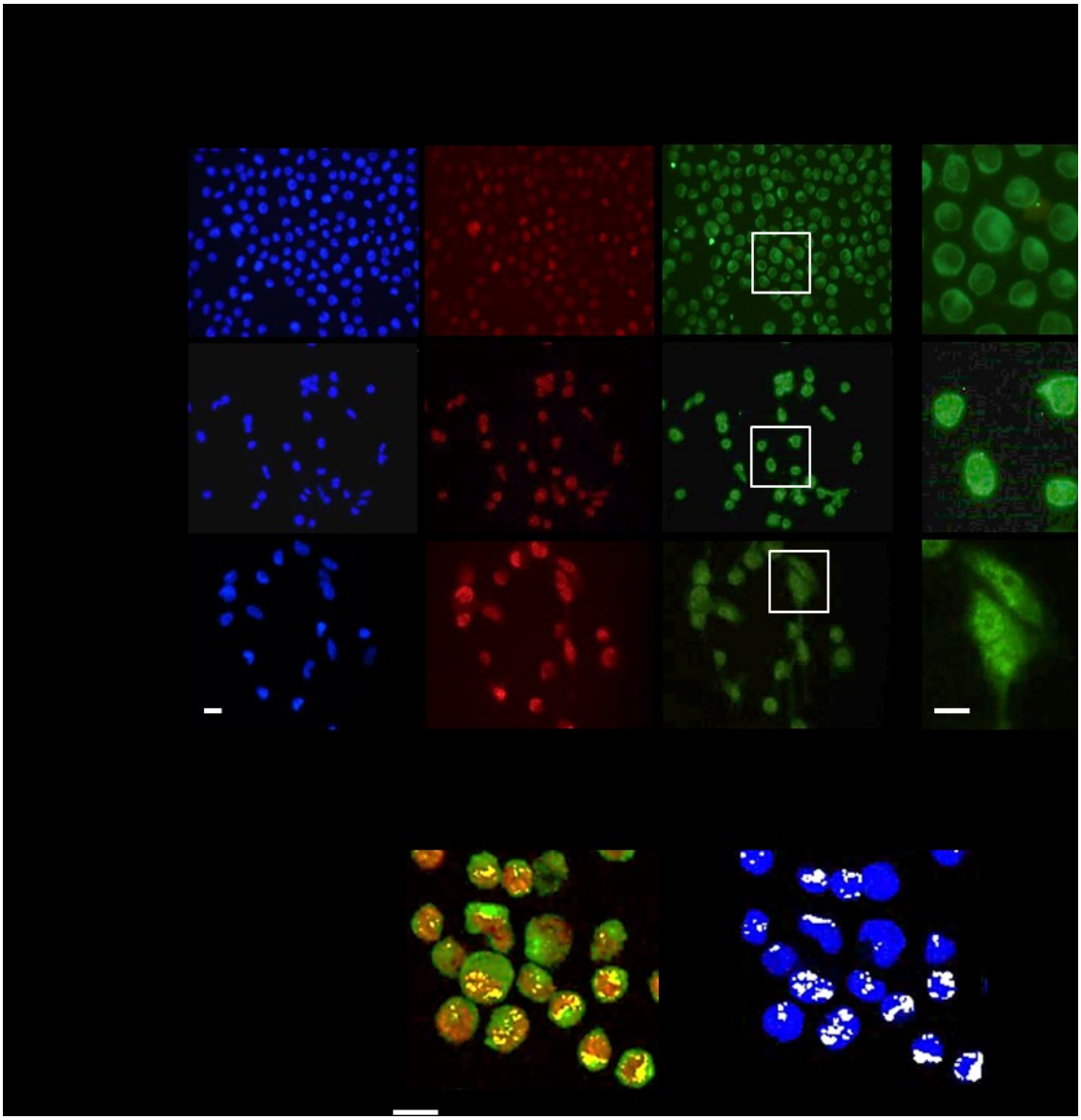

Figure 17. Vav1/PU.1 association in agonist-treated Kasumi-1 cells. (A) Representative fluorescence microscopy images of Kasumi-1 cells treated with ATRA or PMA for 3 days and subjected to immunocytochemical analysis with both anti-PU.1 and anti-Vav1 antibodies. Nuclei were stained with 4,6-diamino-2-phenylindole (DAPI). A higher magnification of Vav1 stained cells is shown on the right. Bar = 20 µm. (B) Representative confocal immunofluorescence images of Kasumi-1 cells treated with PMA and stained simultaneously with antibodies against Vav1 (green staining) and PU.1 (red staining). The nucleus was counterstained in blue by using the TO-PRO®-3 Stain. Merged staining of PU.1 and Vav1 is shown with co-localization resulting in yellow. To the right, PU.1/Vav1 co-localization points were white colored and overlapped to the nuclear staining (blue). Bar = 20 µm.

2. Vav1 regulates the miR-29b/Akt2 axis in breast cancer-derived cells

On the basis of our results in AML derived cells, indicating for Vav1 a role in the expression of miR-29b, the second part of the thesis was aimed to assess if this event also occurs in breast cancer derived cells, in which miR-29b acts as a tumor suppressor (Wang, 2017) and constitutes a potential positive marker for recurrence and metastasis in breast cancer patients (Shinden, 2015).

2.1 Vav1 up-modulates miR-29b in MDA-MB-231 cells

We firstly evaluated the relationship between Vav1 and miR-29b expression in invasive breast cancer tissues included in a well-characterized cohort (TCGA: n=918). As reported in Figure 18, both contributors to the mature miRNA (miR-29b1 and miR-29b2) showed a significant positive correlation with Vav1, suggestive of a role of the protein in promoting the expression of miR-29b.

		VAV1	hsa-mir-29b-1	hsa-mir-29b-2
VAV1	Pearson's r	—		
	p-value	—		
	Spearman's rho	—		
	p-value	—		
hsa-mir-29b-1	Pearson's r	0.247***	—	
	p-value	< .001	—	
	Spearman's rho	0.264***	—	
	p-value	< .001	—	
hsa-mir-29b-2	Pearson's r	0.263***	0.987***	—
	p-value	< .001	< .001	—
	Spearman's rho	0.289***	0.989***	—
	p-value	< .001	< .001	—

* p < .05, ** p < .01, *** p < .001

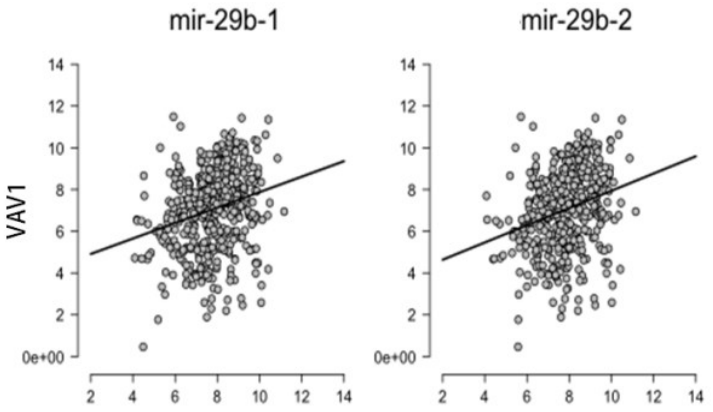

Figure 18. Correlation between Vav1 and miR-29b in invasive breast cancers tissues. The TGCA cohort (n=918) was used and the values were expressed as LOG2 RPM. ***P <0.001.

In order to assess the possible role of Vav1 in regulating miR-29b in breast cancer cells, we used the MDA-MB-231 cell line, highly invasive and poorly differentiated triple-negative breast cancer (TNBC)-derived cells. In particular, we used two MDA-MB-231-derived clones in which Vav1 was stably over-expressed or down-modulated, as described in the Material and Methods section.

The analysis of miR-29b in cells expressing substantially different levels of Vav1 (Figure 19) showed a significantly higher amount of the miRNA in cells over-expressing Vav1 (Figure 20). At variance, lower expression of miR-29b was observed in MDA-MB-231 cells silenced for Vav1 (Figure 20), confirming in this cell model the positive Vav1/miR-29b correlation found in breast cancer tumors.

Once established that Vav1 positively correlates with miR-29b in stably transfected MDA-MB-231 cells, we performed transient transfections experiments, whose results perfectly overlapped the data with clones, confirming the role of Vav1 in modulating the miRNA expression in breast tumor derived cells (data not shown).

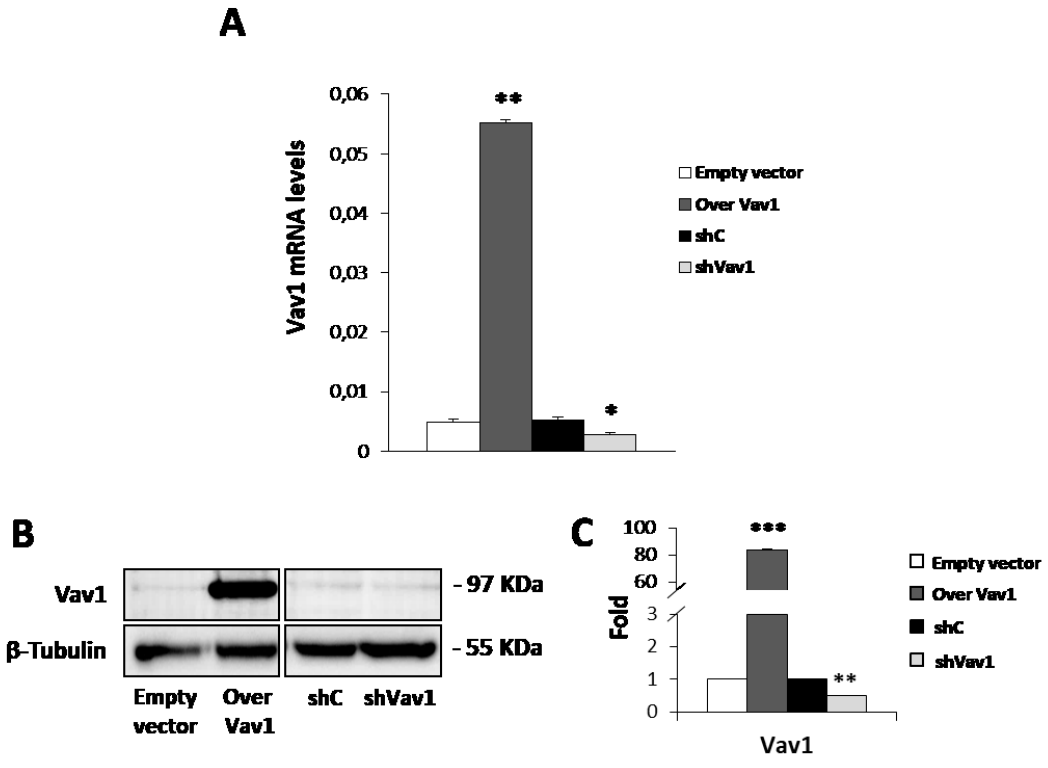

Figure 19. Modulation of Vav1 expression in MDA-MB-231 cells. (A) qRT-PCR analysis of Vav1 mRNA levels in MDA-MB-231 cells in which Vav1 was stably over-expressed (Over Vav1) or down modulated (shVav1). The values were obtained by using the $2^{-\Delta CT}$ method and represent the means of 3 separate experiments ± SD. (B) Representative Western blot analysis using anti-Vav1 antibodies of MDA-MB-231 cells in which Vav1 was stably over-expressed or down modulated. (C) Relative amounts of Vav1 as deduced from densitometry of Western blot bands normalized with β-Tubulin. The mean of 3 separate experiments ± SD is reported. *P <0.05, **P <0.01, ***P <0.001 compared to mock transfected cells.

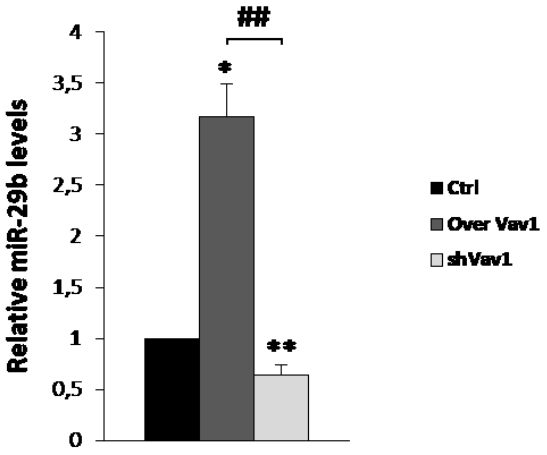

Figure 20. Vav1-related miR-29b in MDA-MB-231 cells. qRT-PCR analysis of miR-29b levels in MDA-MB-231 cells in which Vav1 was stably over-expressed (Over Vav1) or down modulated (shVav1). The values, obtained by using the 2-ΔΔCT method, are shown as fold changes relative to the mock transfected cells, and represent the means of 3 separate experiments ± SD. *P <0.05, **P <0.01 compared to control conditions (Ctrl). ##P <0.01.

In order to understand the mechanism involved in the Vav1 related modulation of miR-29b, we looked for a transcription factor cooperating with Vav1, as we have already demonstrated in AML derived cells.

Since, as above reported, we have found that PU.1 is the transcription factor involved with Vav1 in the regulation of miR-29b in AML-derived cells, a first investigation was aimed to establish if the same mechanism is present in breast cancer-derived cells.

Immunochemical analysis with a specific antibody confirmed the expression of PU.1 in MDA-MB-231 and revealed that its level is independent on Vav1 amount (Figure 21).

At variance to what we have found in AML-derived cells, the ChIP analysis of PU.1 association to its potential binding regions on both miR-29b promoters on Chromosome 1 and Chromosome 7 failed to reveal protein associated DNA (data not shown).

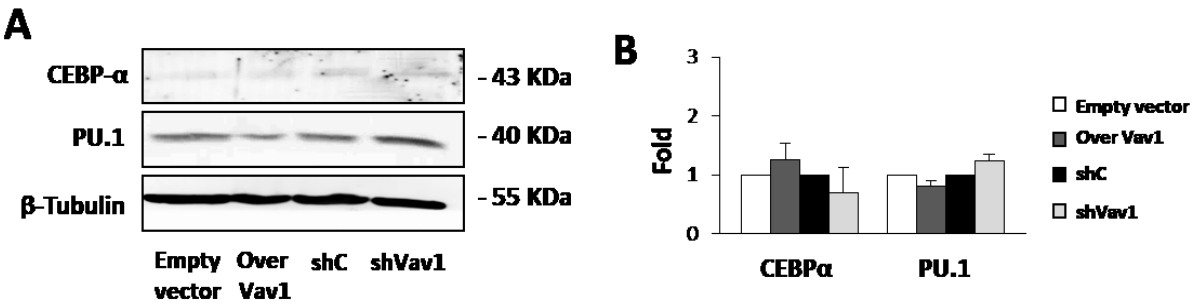

Figure 21. Vav1 related CEBPα and PU.1 amount in MDA-MB-231. (A) Representative Western blot analysis using antibodies anti-PU.1 and CEBPα antibodies of MDA-MB-231 cells in which Vav1 was stably over-expressed (Over Vav1) or down modulated (shVav1). (B) Relative amounts of CEBPα and PU.1 as deduced from densitometry of Western blot bands normalized with β-Tubulin. The mean expression levels of 3 separate experiments ± SD are reported.

As CEBPα is the transcription factor mainly responsible of miR-29b regulation in leukemic cells (Eyholzer, 2010), a further set of experiment was aimed to assess the role of CEBPα in the Vav1-related regulation of the miRNA in MDA-MB-231 cells.

Despite the amount of CEBPα was not correlated with the levels of Vav1 and of miR-29b in our breast cancer cells model (Figure 21), by performing ChIP assays we revealed the recruitment of CEBPα to the miR-29b promoter on Chromosome 7, that significantly increased in cells over-expressing Vav1 (Figure 22). On the contrary, a significantly lower amount of DNA associated to CEBPα was found in cells expressing low Vav1 (Figure 22).

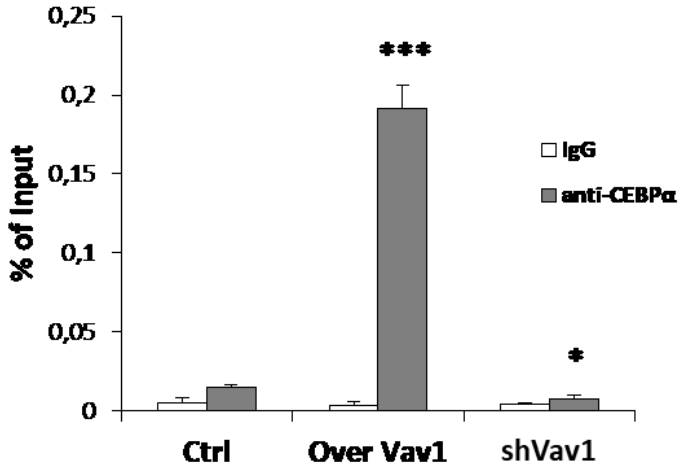

Figure 22. *In vivo* interaction of CEBPα with miR-29b promoter in MDA-MB-231. Analysis of in vivo recruitment of CEBPα to miR-29b promoter on Chr 7 performed by chromatin immunoprecipitation with an antibody directed against CEBPα in MDA-MB-231 cells in which Vav1 was stably over-expressed (Over Vav1) or down modulated (shVav1). The data are shown as percentage of the Input (genomic DNA collected before immunoprecipitation). IgG: negative control. Values represent the means of 3 separate experiments ± SD. *P <0.05, ***P<0.001 compared to mock transfected cells.

This first bulk of data allowed to assess that CEBPα is directly involved in the regulation of mR-29b in breast cancer derived cells and that an adequate amount of Vav1 is relevant for the access of the transcription factor to its DNA consensus region.

To better explore the role of Vav1 in the expression of miR-29b in our cell model, we also evaluated its effects on the known precursors of mature miRNA. For this purpose, specific primers were used for pri-miR-29b1, pri-miR-29b2 that amplify a region flanking the Drosha cleavage site (Anastasiadou, 2010). Specific primers have been designed for pre-miR-29b1 and pre-miR-29b2 and the levels of all precursors were evaluated through qRT-PCR in MDA-MB-231 cells expressing different Vav1 amounts. As reported in Figure 23, the pri-miR-29b1 is the most expressed precursor, according with the preferential interaction of CEBPα with promoter on Chromosome 7. The significantly lower expression

of pri-miR-29b1 in cells with low Vav1 might reflect the reduced transcriptional activity, according with the ChIP data. On the other hand, the apparent lower (or not increased) level of pri-miR-29b1 in cells over-expressing Vav1 and showing high miR-29b suggests that Vav1 could be also involved in the processing of the precursors, regulating miR-29b at both the transcriptional and post-transcriptional level.

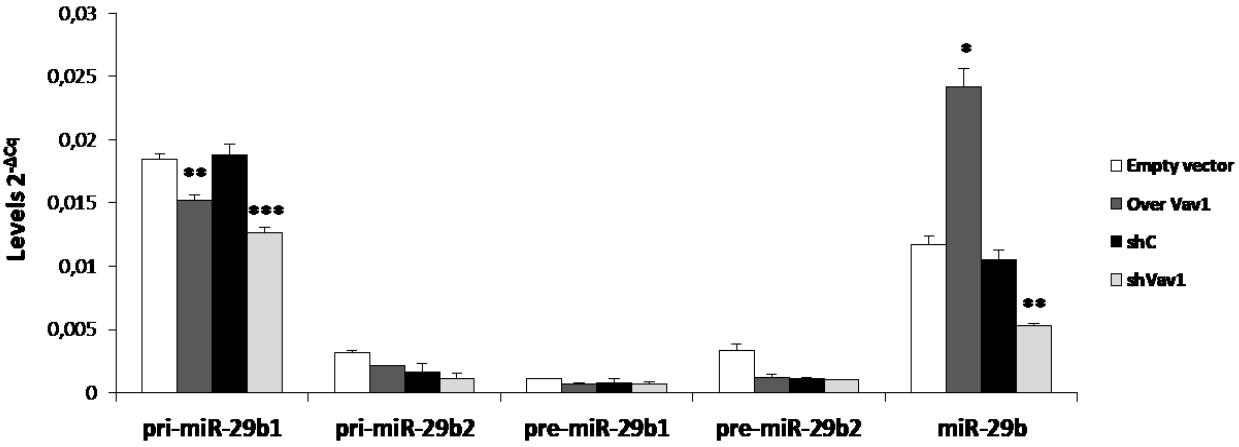

Figure 23. Vav1 involvement in miR-29b biogenesis. qRT-PCR analysis of pri-miR-29b1/2 and pre-miR-29b1/2 levels in MDA-MB-231 cells in which Vav1 was stably over-expressed (Over Vav1) or down modulated (shVav1). The values were obtained by using the $2^{-\Delta CT}$ method and represent the means of 3 separate experiments ± SD. *P <0.05, **P <0.01 ***P<0.001 compared to mock transfected cells

2.2 The Vav1/miR-29b pathway down-modulates the expression of Akt2 in breast tumor cells

On the basis of our previous results indicating that, in mammary tumor cells, Vav1 regulates the expression of specific Akt isoforms (Grassilli, 2018), in turn targets of miR-29b in different tumor models (Teng, 2015), a further set of experiments was aimed to establishing the existence of a Vav1/miR-29b/Akt axis in MDA-MB-231 cells.

We first tried to establish if miR-29b modulates the expression of the Akt2 and Akt3 isoforms of Akt, known to be direct targets of the miRNA in ovarian cancer (Teng, 2015).

With this aim, MDA-MB-231 cells were then transfected with synthetic miR-29b mimic and inhibitor, that resulted very effective in up-modulating or silencing the expression of the miRNA (Figure 24A).

As we reported in Figure 24B, miR-29b mimic was able to down-modulate the recovery of mRNA for Akt2 while was ineffective on mRNA for Akt3.

The decrease of Akt2 as a consequence of mimic administration was confirmed by immunochemical analysis (Figure 25).

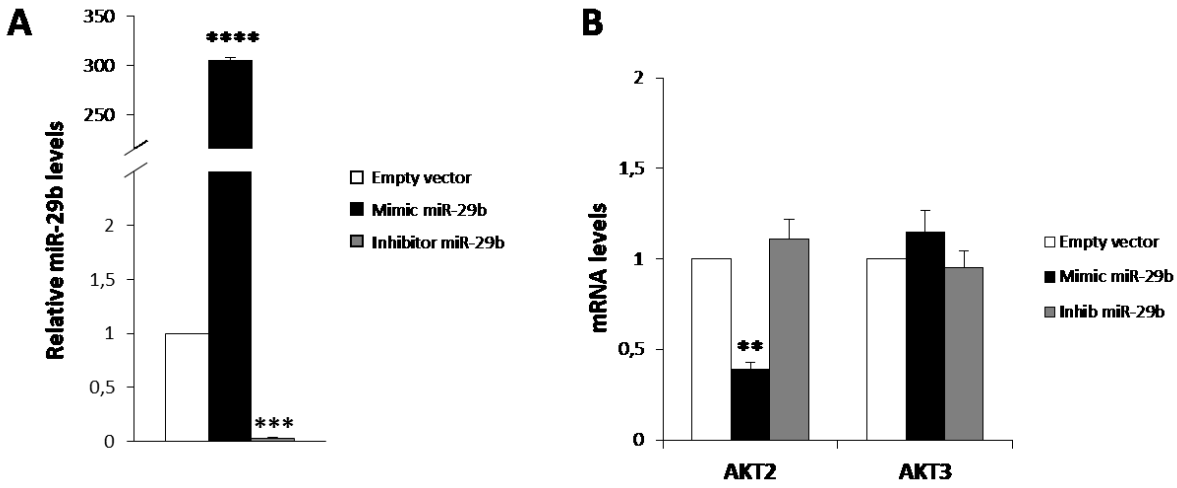

Figure 24. Modulation of miR-29b levels in MDA-MB-231 cells. qRT-PCR analysis of miR-29b (A) levels and of mRNA for Akt2 and Akt3 (B) in MDA-MB-231 cells in which miR-29b was up-modulated (mimic) or down modulated (inhibitor). The values were obtained by using the $2^{-\Delta\Delta CT}$ method, are shown as fold changes relative to the control condition (empty vector), and represent the means of 3 separate experiments ± SD. **P <0.01 ***P<0.001 compared control condition (empty vector).

In order to verify if the Vav1–related modulation of miR-29b effectively results in the modulation of Akt2 expression, we analyzed the level of this Akt isoform in MDA-MB-231 stably expressing different Vav1 levels. As shown in Figure 26A, mRNA for Akt2 is significantly higher in cells in which Vav1 was silenced and the use of a specific antibody revealed an higher amount of the protein (Figure 26B, 26C).

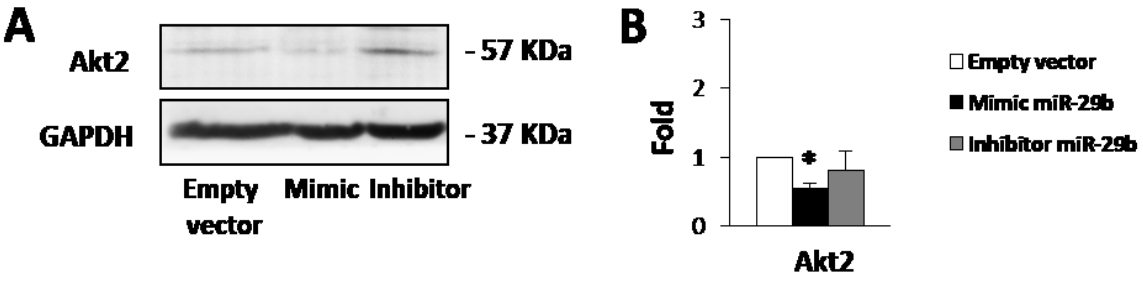

Figure 25. miR-29b related levels of Akt2 and Akt3 in MDA-MB-231 modulated by. (A) Representative Western blot analysis using an antibody against Akt2 of MDA-MB-231 cells in which miR-29b was up-modulated (mimic) or down modulated (inhibitor). (B) Relative amounts of Akt2 as deduced from densitometry of Western blot bands normalized with GAPDH. The mean expression levels of 3 separate experiments ± SD is reported. *P <0.05 compared to control condition (empty vector).

Accordingly, a decrease of Akt2 was revealed in cells over-expressing Vav1 (Figure 26B, 26C). The evaluation of mRNA for Akt3 failed to show difference in cells expressing different levels of Vav1 (Figure 26A), confirming our previous data on the same cell

model (Grassilli, 2018) and corroborating the Vav1/miR-29b/Akt2 relationship in this cell model.

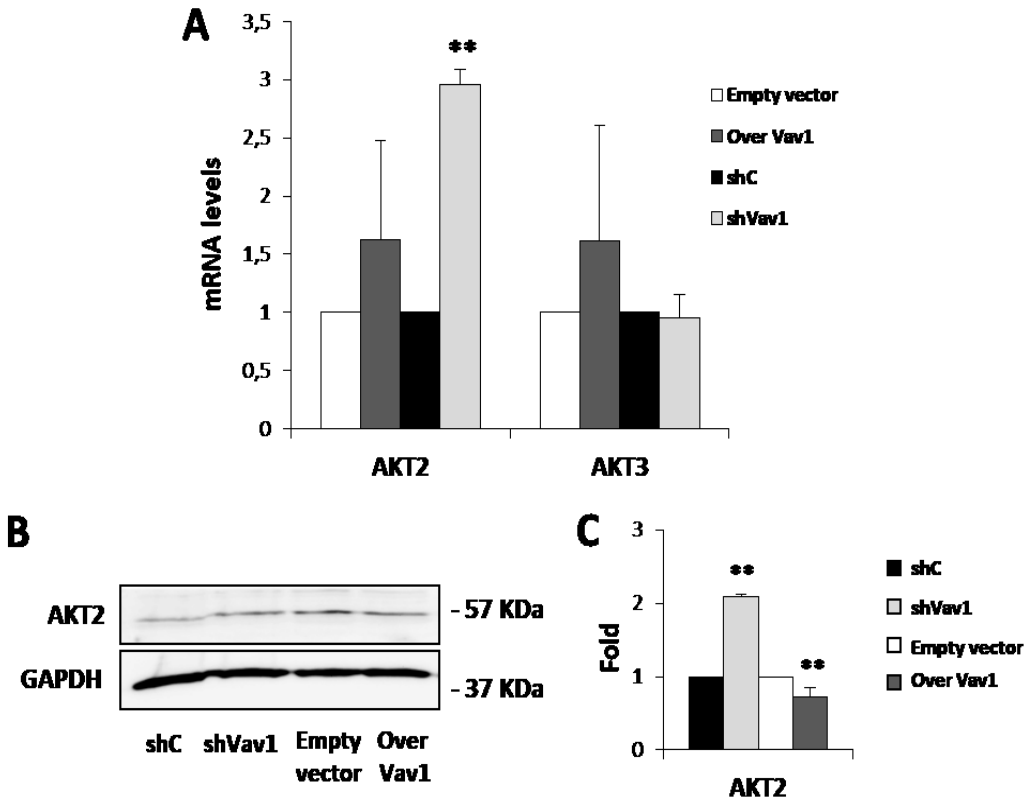

Fig.26 Akt2 levels in MDA-MB-231. (A) qRT-PCR analysis of Akt2 mRNA levels in MDA-MB-231 cells in which Vav1 was over-expressed or down modulated. The values were obtained by using the 2-ΔΔCT method, are shown as fold changes relative to the untreated condition, and represent the means of 3 separate experiments ± SD. **P <0.01 compared to untreated conditions. (B) Representative Western blot analysis using antibodies anti-Akt2 of MDA-MB-231 cells in which Vav1 was over-expressed or down modulated and relative amounts of Akt2 as deduced from densitometry of Western blot bands normalized with GAPDH. The mean expression levels of 3 separate experiments ± SD. **P <0.01 compared to control condition.

Discussion

Dysregulation of members of the miR-29 family has been reported in various cancers including leukemia (Garzon, 2009) and breast cancers (Melo, 2011). In particular, miR-29b was reported to act as a tumor suppressor targeting multiple oncogenes such as DNA methyltransferase 3A/B (DNMT3A/DNMT3B) that induces DNA hypomethylation in both tumors (Garzon, 2009; Sandhu, 2013). In order to identify drugs able to restore the expression of miR-29b, generally down-modulated in tumors, the machinery that regulates the expression of this miRNA was explored in cells derived from both acute myeloid leukemia (AML) and invasive breast cancer.

AML is predominantly a disease of older adults associated, also in case of karyotypes related to a favorable prognosis, (Kadia, 2015; De Kouchkovsky, 2016) with poor long-term outcomes with available therapies. In the last years, hypomethylating agents, traditionally employed in treatment of myelodysplastic syndrome, have shown efficacy in patients with non-APL myeloid leukemia (Gardin, 2017). The level of miR-29b seems to be important in the response to hypomethylating agents, suggesting that strategies designed to increase this miRNA could effectively improve the prognosis of AML patients.

At present, the absence of clinical application of delivery systems developed for synthetic miR-29b in animal models (Huang, 2013) makes the activation of its transcription the only efficient strategy for restoring the miRNA level in patients with myeloid leukemia. As transcription factors responsible for miR-29b expression are generally deregulated in AML, (Amodio, 2015; Yan, 2015; Wang, 2010) the first part of this study was aimed to identify molecules with the ability to restore the miR-29b transcriptional machinery. On the basis of recent findings demonstrating that PU.1 is a transcriptional regulator of the miR-29b2/c locus in APL-derived cells (Batliner, 2012), we firstly investigated the ability of this transcription factor to modulate miR-29b in non-APL cells. As experimental model we choose the myeloid-derived Kasumi-1 cells, displaying the t(8;21) chromosomal rearrangement, the most common cytogenetic subtype of AML, whose survival rate is 30% on a 5-year basis (Gardin, 2017). The notion that overexpression of PU.1 in Kasumi-1 overcomes its functional block induced by the fusion protein AML1-ETO, in turn involved in a regulatory circuit with miR-29b (Zaidi, 2017), makes this cell line a suitable model to correlate the activity of PU.1 to the miRNA level.

Considering its role on miR-29b transcription in NB4 cells, Kasumi-1 were firstly treated with ATRA, which even though induced PU.1 expression, failed to promote the expression of miR-29b in this cell model. When we treated Kasumi-1 with PMA, known to induce

phosphorylation and nuclear translocation of PU.1 (Park, 2016), we revealed the up-modulation of the transcription factor accompanied by a substantial increase in miR-29b, indicating that the sole agonist-induced overexpression of PU.1 is not sufficient to activate the transcriptional machinery at the miR-29b promoters.

To understand the rational of the agonist-related effects of PU.1 on miR-29b expression, we investigated the *in vivo* binding of PU.1 to miRNA promoters taking into account our previous data demonstrating that, in APL-derived cells treated with ATRA, some PU.1 activities are correlated with the nuclear amount of Vav1, a multidomain protein variously involved in gene expression and mRNA processing (Bertagnolo, 2012, Grassilli, 2014). We firstly investigated this issue in APL-derived NB4 cells, in which we found that, similarly to what observed for regulation of CD11b and miR-142-3p (Brugnoli, 2010; Grassilli, 2016), the interaction of PU.1 with both miR-29b promoters is entirely dependent on adequate levels of Vav1. In this cell model, Vav1 is not present in the PU.1-containing molecular complexes on miR-29b promoters but colocalizes with the transcription factor inside the nuclear compartment, confirming that a nuclear PU.1/Vav1 cooperation is necessary to regulate the agonist-induced expression of miR-29b in APL-derived cells.

The same analysis performed in Kasumi-1 cells revealed another scenario. In fact, we found that also in this cell model adequate levels of Vav1 are necessary for the expression of miR-29b while, at variance with NB4 cells, this protein is not necessary for the interaction of PU.1 with the miRNA promoter. Interestingly, in Kasumi-1, in contrast to NB4, we found Vav1 in the molecular complexes on the consensus region for PU.1 on miR-29b promoter, demonstrating for the first time the direct participation of Vav1 to the transcriptional machinery in non-APL cells.

Once we demonstrated that the cooperation between PU.1 and Vav1 is essential for the expression of miR-29b in both NB4 and Kasumi-1 cells, we tried to identify the events that prevented the induction of miR-29b in ATRA-treated Kasumi-1, in which both PU.1 and Vav1 were up-modulated by the agonist. The analysis of the intracellular distribution of the two proteins suggested that the PU.1/Vav1 association inside the nuclear compartment is a crucial event for inducing up-modulation of miR-29b.

This first bulk of data allow to conclude that the expression of miR-29b in myeloid leukemia-derived cells is almost completely dependent on Vav1, that plays peculiar roles in APL- and non-APL–derived cell lines. These results add new information about the transcriptional machinery that regulates miR-29b expression in AML-derived cells and may help in identifying drugs useful in pre-treatments of non-APL leukemia that take advantage from therapies based on hypomethylating agents.

On the basis of the results obtained in AML-derived cells, the possible involvement of Vav1 in regulating miR-29b was the explored in breast tumors. Our interest was mainly focused on triple-negative breast cancer (TNBC), the most aggressive breast cancer subtype with a high propensity for metastasis and a poor prognosis (Foulkes, 2010), due to the lack of targeted therapies (von Minckwitz, 2012; Heitz, 2009). Several research on miRNA functions show that they can act as either oncogenes or tumor suppressors in breast cancer and more specifically in TNBC (Yang, 2015; van Schooneveld, 2015; Gyparaki, 2014), suggesting that they could be therapeutic targets for this tumor subtype (Ghelani, 2012). Among the miRNA de-regulated in breast tumors, miR-29b1 is down-modulated in both TNBC tissues and cell lines, in which impacts on multiple oncogenic features and renewal potential (Drago-Ferrante 2017). Despite their possible role as therapeutic targets against TNBC, and although miRNA biogenesis and normal or deregulated expression have been extensively described (Jansson, 2012), the mechanisms leading to their abnormal expression in cancer remain largely ununderstood. In this context, a better knowledge of the machinery that regulates the expression of miR-29b in breast tumor cells could constitute an important contribution to the treatment of highly invasive breast cancers.

On the basis of the results obtained in AML-derived cells, demonstrating that adequate levels of Vav1 are necessary for the expression of miR-29b, the relationship between the two molecules was firstly evaluated in invasive breast cancer tissues. A positive correlation was found, suggestive for a role of Vav1 in promoting miR-29b expression in breast tumors. To assess the effective involvement of Vav1 in the miR-29b expression, we performed *in vitro* experiments with the highly invasive MDA-MB-231 cell line, the most commonly used TNBC-derived cell line. In particular we used two MDA-MB-231-derived clones in which Vav1 was stably over-expressed or down-modulated.

The analysis of miR-29b in cells expressing different levels of Vav1 confirmed in this cell model the positive Vav1/miR-29b correlation found in breast cancer tissues. Because in AML-derived cells we demonstrated that Vav1 is involved in gene expression by direct interaction with or as a facilitator of transcription factors, we explored the role of Vav1 in the transcription process of miR-29b in breast tumor cell. On the basis of the results on AML-derived cells, we firstly investigated the possible cooperation of Vav1 with PU.1. Our results, even if confirmed the presence of PU.1 in MDA-MB-231 cells, failed to reveal association of the transcription factor with its potential binding regions on both miR-29b promoters on Chromosome 1 and Chromosome 7. At variance, when we investigated CEBPα, the transcription factor mainly responsible of miR-29b regulation in leukemic

cells (Eyholzer, 2010), we revealed its recruitment to the binding region on the miR-29b promoter, which significantly increased in cells over-expressing Vav1 and decreased in cells in which Vav1 was silenced. These data allow us to affirm that, despite CEBPα expression is down-regulated in breast carcinomas (Gery, 2005) and its amount is apparently unrelated to the levels of Vav1 and of miR-29b in our breast cancer cells model, it is directly involved in the regulation of mR-29b in a Vav1-dependent manner.

The implication of Vav1 in miR-29b expression was further investigated by performing the analysis of the miRNA precursors. The pri-miR-29b1 resulted the most expressed precursor, according with the interaction of CEBPα with promoter on Chromosome 7. The relationship between its amount and the levels of Vav1 suggested that this protein could also be involved in the processing of pri-miR-29b1, regulating miR-29b at both transcriptional and post-transcriptional level.

The last part of our study was focused on the Akt pathway, stemming from the notion that genes for Akt2 and Akt3 were identified as potential downstream target of miR-29b (Teng, 2015) and that we previously demonstrated that Vav1 down-modulates Akt in breast cancer cell lines with different phenotypes (Grassilli, 2018). We revealed that silencing of Vav1 in MDA-MB-231 correlates with low levels of Akt2, confirming our previous data on the same cells model level. This event seems to depend on the Vav1-related decrease of miR-29b levels, as confirmed by the use of specific inhibitor of the miRNA. The decrease of Akt2 induced by up-modulation of miR-29b definitively assessed the existence of a Vav1/miR-29b/Akt2 axis in this cell model of triple negative breast tumor. On the basis of our results we can conclude that strategies aimed to increase Vav1 could be useful for tumors with a triple-negative phenotype, for which the development of Akt inhibitors is particularly problematic and target-based therapies are not currently available.

Overall, the data reported in this thesis add substantial new information about the regulation of miR-29b expression in both myeloid leukemia and breast cancer and identify Vav1 as a crucial positive modulator of this tumor suppressor miRNA. Our results can help in identifying drugs that, up-modulating the Vav1/miR-29b axis, may be useful in the treatment of leukemia and solid tumors.

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