Published OnlineFirst August 23, 2016; DOI: 10.1158/1541-7786.MCR-16-0164

Review

Molecular Cancer Research

Rnd3 in Cancer: A Review of the Evidence for Tumor Promoter or Suppressor

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Abstract

Rho-GTPases are members of the Ras superfamily of small GTPases and are general modulators of important cellular processes in tumor biology such as migration and proliferation. Among these proteins, Rnd3/RhoE, an atypical Rho-GTPase devoid of GTP hydrolytic activity, has recently been studied for its putative role in tumorigenesis. Indeed, Rnd3 is implicated in processes, such as proliferation and migration, whose deregulation is linked to cancer development and metastasis. The aim of this review is to provide an overview of the data surrounding Rnd3 deregulation in cancers, its origin, and consequences. Presented here is a comprehensive account of the expression status and biological output obtained in prostate, liver, stomach, colon, lung, and brain cancers as well as in melanoma and squamous cell carcinoma. Although there appears to be no general consensus about Rnd3 expression in cancers as this protein is differently altered according to the tumor context, these alterations overwhelmingly favor a protumorigenic role. Thus, depending on the tumor type, it may behave either as a tumor suppressor or as a tumor promoter. Importantly, the deregulation of Rnd3, in most cases, is linked to patient poor outcome.

Implications: Rnd3 has prognostic marker potential as exemplified in lung cancers and Rnd3 or Rnd3-associated signaling pathways may represent a new putative therapeutic target. *Mol Cancer Res; 14(11); 1033–44. ©2016 AACR.*

Introduction

Small GTP-binding proteins are signaling components that are important in converting extracellular stimuli into a wide range of cellular responses. Rho-GTPases, which are part of the Ras superfamily of GTPases, control cell adhesion, cell-cycle progression, cell migration, cell morphogenesis, gene expression, and actin cytoskeleton dynamics. Therefore, they play critical roles in tumor biology (1, 2). Rho-GTPases are indeed important regulators of tumor cell proliferation, survival, and invasion. Rho-GTPases were recently involved in the interaction of cancer cells with the microenvironment, including cancer-associated fibroblasts, endothelial, and immune cells (1). In addition, through the regulation of gene expression, Rho-GTPases control angiogenesis, stemness, and immune response. Thus, many of the "hallmarks of cancer" as defined by Hanahan and Weinberg (3) are linked to the deregulation of Rho-GTPases (4). Genetic alterations of small GTPases underscore their important role. However, for a long time, in contrast with Ras, no gain- or loss-of-function mutations were found in Rho-GTPases in solid tumors. Recently, large-scale exome sequencing of tumors led to the discovery of recurrent mutations of Rho-GTPase *RHOA* and *RAC1* genes (5–10). In addition, the functions of most Rho-GTPases are perturbed in cancers through abnormal expression or altered regulation of their activities. This is the case of Rnd3/RhoE, an atypical member of the Rho family, which is not, so far, described as recurrently mutated in tumors, but which expression is altered in an increasing number of cancers. Puzzlingly, depending on the cancer type, Rnd3 has been considered either as an oncogene or a tumor suppressor. Thus, the aim of this review is to provide an overview of the data describing the de-regulation of Rnd3 in cancers and its consequences.

Rnd3/RhoE, an Atypical Rho-GTPase Member

Besides the well-known triad, Cdc42, Rac1, and RhoA, the Rho-GTPase family harbors 20 members. Rnd3/Rho8/RhoE belongs to the Rnd sub-family that also comprises the closely related Rnd1/Rho6 and Rnd2/Rho7/RhoN proteins, which share 54% to 63% identity pairwise (11). Evolutionary history of the Rho family revealed that Rnd members emerged in chordates, suggesting that they evolved relatively recently (12). Rnd proteins are atypical Rho-GTPases compared to their typical partners in terms of the proteins' structures and functions (13). First, Rnd1 and Rnd3 have N- and C-terminal extensions of about 30 amino acids relative to Cdc42 (ref. 11; Fig. 1). Then, unlike the best-characterized Rho family members that are posttranslationally modified by addition of a geranyl-geranyl group (20 carbons lipid motif) at the C-terminal CAAX (C = Cys, A = aliphatic, X = any amino acid) tetrapeptide motif, Rnd proteins are predominantly

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doi: 10.1158/1541-7786.MCR-16-0164

Published OnlineFirst August 23, 2016; DOI: 10.1158/1541-7786.MCR-16-0164

Figure 1.

Alignment of Rnd1, 2, and 3 compared with RhoA, Rac1, and Cdc42. The regions binding the guanine nucleotide, the region considered as the effector binding site, and the C-terminal motif for prenylation (CAAX) are boxed. Stars over the sequence indicate the positions corresponding to key amino acids required for GTP hydrolysis. These data are available from the Protein NCBI database under accession numbers NP_001655 (RhoA), NP_008839 (Rac1), NP_001034891 (Cdc42), NP_055285 (Rnd1), NP_005431 (Rnd2), and NP_005159 (Rnd3/RhoE). The amino acid sequences of human Rho-GTPases were aligned using the Kalign (2.0) program for the CLUSTAL multiple sequence alignment.

farnesylated (15 carbons lipid motif). Finally, unlike canonical members of the Rho family that are regulated by switching between an inactive GDP-bound and an active GTP-bound form, Rnd proteins are constitutively bound to GTP. Indeed, as these proteins lack the amino acids that are critical for GTP hydrolysis (Fig. 1), they have no detectable GTPase activity. Accordingly, in its basal state Rnd3 is not cytosolic, but is predominantly associated with membranes (14). As a consequence, regulation of Rnd3 is controlled preferentially by the balance between transcription/translation and degradation, and/or by posttranslational modifications such as phosphorylation, rather than by classical Rho regulators such as guanine nucleotide exchange factors (GEF), GTPase-activating proteins (GAP), and GDP-dissociation inhibitors (GDI; refs. 15, 16). Indeed, Rnd3 was also found regulated through interactions with other proteins, such as 14-3-3 (17) or effectors as Syx/PLEKHG5 (18). Despite its atypical regulation, Rnd3 has been implicated in functions commonly regulated by Rho-GTPases such as the remodeling of the actin cytoskeleton, and in many basic cellular processes such as cell proliferation, differentiation, survival, motility, and adhesion (Fig. 2). In human adult, Rnd3 is widely expressed with the lowest expression in brain, kidney, and pancreas (11, 14). However, it is quite different expression profile among mouse tissues as an enriched expression of Rnd3 was found in lungs and brain (19). The first clues for the *in vivo* function of Rnd3 came with the development of knockout (KO) mice that lack Rnd3 expression. Rnd3 null mice died either at E11.0 (C57BL/6 background) or shortly after

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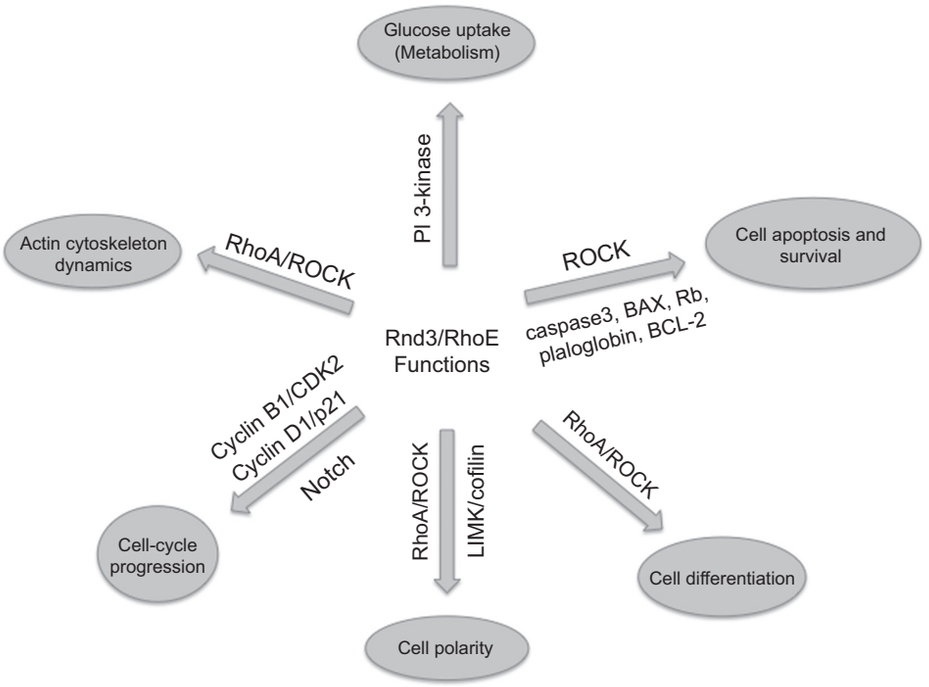

Figure 2.
Tumor functions of Rnd3/RhoE. Rnd3 has been found to regulate different cellular processes important for tumor growth and progression *in vitro*. Indicated are major proteins implicated in the specific functions.

birth (hybrid 129SvEvBrd and C57BL/6J background) showing that, depending of the genetic background, Rnd3 is essential either for embryo or postnatal development (20, 21). In the C57BL/6 background, death of Rnd3 null embryos was attributed to fetal arrhythmias and heart failure (21). Rnd3 haploinsufficient (Rnd3±) mice, which are viable, yet developed dilated cardiomyopathy with heart failure, and the study revealed that Rnd3 acted as a proangiogenic factor involved in cardiac responsive angiogenesis through HIF1α–VEGFA signaling (22). However, Rnd3 ablation in the mixed background results in neuromotor impairment and neurodevelopmental delay, demonstrating a defect in the development of the nervous system (20). This has been confirmed *in vitro* using Rnd3-deficient hippocampal neurons that display a decrease in neurite and axon outgrowth and a delay in neuronal polarization (23). These defects were linked to the RhoA/ROCK signaling pathway. Indeed, Rnd3 was early described as a RhoA antagonist and involved in the regulation of actomyosin contractility. In addition to its role described *in vivo* in neurons, Rnd3 was involved in smooth muscle contractility (24, 25) and in osteoclast and cancer cell migration (26–28).

Rnd3 was also shown to regulate the cell-cycle progression, apparently independently of cytoskeleton remodeling (15, 29). Several mechanisms may be involved, depending on the cellular context. For instance, Rnd3 was shown to downregulate cyclin D1 translation *in vitro* in NIH-3T3 cells through decreased 4E-BP1 phosphorylation (30) whereas it regulates Notch signaling in ependymal epithelium, resulting in hydrocephalus through epithelial overgrowth in Rnd3 null mice (19). Thus, these results demonstrated that Rnd3 has pleiotropic effects on cell proliferation.

Thus, *in vivo* and *in vitro* studies have implicated Rnd3 in various functions (ref. 31; Fig. 2) including regulation of actin cytoskeleton dynamics, cell polarity, cell-cycle progression, and apoptosis, mechanisms that, when deregulated, may lead to cancer development or progression.

Rnd3/RhoE Expression Is Differently Altered in Cancers

A growing number of studies have described the alteration of Rnd3 expression and function in tumors. Interestingly, Rnd3 expression is differentially regulated in various cancers. Indeed, *RND3* mRNA and protein are underexpressed in prostate and gastric cancers (32, 33) and overexpressed in non–small cell lung (NSCLC; refs. 34, 35) and pancreatic (36) cancers. Thus, although Rnd3 overexpression is considered as an unfavorable prognostic factor in lung cancer, *RND3* is also considered as a tumor suppressor gene in prostate cancer. To address Rnd3 functions either as tumor suppressor or oncoprotein in specific cell types, studies using organ-specific KO mice will be required. But, an accurate description of the alteration of Rnd3 expression in human tumors is also required. Thus, it is important to make an overview of these deregulations and the way they have been stated. Indeed, the methods of analysis of gene expression, the numbers of studies and cases are key to achieve an exact description in the tumor context. Tables 1 and 2 summarize the data available about the deregulation of Rnd3 in various cancers and below is the description of the results obtained for each cancer type.

Prostate cancer

One of the first studies demonstrating that Rnd3 was deregulated in tumors was published for prostate cancer in 2005 (32). Rnd3 expression was significantly lower in malignant when compared with benign prostate tissue samples (Table 1). In agreement with this study, three microarray data available in Oncomine database, also found a significant underexpression of Rnd3 in prostate carcinoma when compared with normal tissues

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Table 1. Rnd3 deregulation in cancers

Cancer type	Rnd3: up or down?	Nb of tumor/control	Method of detection	Source of deregulation	Output	References
Prostate cancer	Down	20 T/20 NT	qRT-PCR	nd	nd	(Bektic et al., 2005)
Hepatocellular carcinoma	Down	120 T/28 NT 27 paired N/T tissues	qRT-PCR WB, IHC	nd	Association with presence of intrahepatic metastases	(Grise et al., 2012)
Hepatocellular carcinoma	Down	19 paired N/T tissues 99 T/79 NT	qRT-PCR, WB IHC	nd	Association with shorter survival and poor prognosis/independent prognostic marker	(Luo et al., 2012a)
Hepatocellular carcinoma	Down	71 paired N/T tissues 2 paired N/T tissues	qRT-PCR WB	nd	Association with shorter disease-free survival	(Ma et al., 2013)
Colorectal cancer	Down	41 T/49 NT 19 paired N/T tissues	IHC qRT-PCR	miR17 upregulation	nd	(Luo et al., 2012b)
Colorectal cancer	Up	202 paired N/T tissues 6 paired N/T tissues	IHC WB	nd	Correlations with invasion and metastasis/independent prognostic marker	(Zhou et al., 2013)
Gastric cancer	Up	90 paired N/T tissues 40 primary/metastases paired tissues	IHC on tissue array	nd	Correlations with differentiation grade and tumor staging/independent prognostic marker	(Feng et al., 2013)
Gastric cancer	Up	27 paired N/T tissues	qRT-PCR	miRNA-200c upregulation	nd	(Chang et al., 2014)
Non–small cell lung cancer	Up	30 paired N/T tissues	qRT-PCR, IHC, WB	Correlation with DNA copy number changes	Association with smoking history	(Cuiyan et al., 2007)
Non–small cell lung cancer	Up	115 T	IHC on tissue array	nd	Association with shorter survival/independent prognostic marker	(Zhang et al., 2007)
Non–small cell lung cancer	Up	107 T	Micro-array	nd	Part of a 4-gene signature that predicts overall and disease-free survival	(Raz et al., 2008)
Non–small cell lung cancer	Up	361 T (training cohort) 433 T (validation cohort) 1006 T (validation cohort)	qRT-PCR	nd	Part of a 14-gene signature that identifies patients at high risk for mortality after surgical resection (patent US20130059747, patent EP2726635A1)	(Kratz et al., 2012)
Glioblastoma	Down	15 T/15 normal brain tissues 13 T/13 normal brain tissues 4 paired N/T tissues	IHC qRT-PCR WB	Notch signaling	Correlation with patient survival time, tumor size, and tumor cell proliferation	(Liu et al., 2015)
Glioblastoma	Up	45 grade II/81 grade IV gliomas 50 grade II/24 grade IV gliomas (validation) 2 grade II/2 grade III/2 grade IV gliomas 2 grade II/1 grade III/2 grade IV gliomas	Micro-array Micro-array WB IHC	nd	Association with grade	(Clarke et al., 2015)
Glioblastoma	Up	12 paired N/T tissues	qRT-PCR	miR128 downregulation	nd	(Shang et al., 2016)
Glioma	Up	48 paired N/T tissues				
Subependymal giant cell astrocytomas	Up	10 T/5 NT	qRT-PCR	mTOR activity due to mutations in TCS1 and 2 gene	Benign phenotype and low invasiveness	(Tyburczy et al., 2010)
Esophageal squamous cell carcinoma	Down	128 T	IHC, ISH	nd	Association with differentiation degree, clinical staging and lymph node metastasis	(Zhao et al., 2012)
Oral or skin squamous cell carcinoma	Down	23 paired N/T tissues	qRT-PCR	Notch1 downexpression	Suppression of the differentiation potential	(Zhu et al., 2014)

Abbreviations: IHC, immunohistochemistry; ISH, *in situ* hybridization; nd, not determined; paired N/T tissues, paired nontumor versus tumor tissues; qRT-PCR, quantitative real-time PCR; WB, Western blot analysis.

(37–39). In addition, prostatic carcinoma cell lines showed lower amount of Rnd3 protein than primary human prostate epithelial cells (32). These data suggested that Rnd3 is constitutively expressed in the benign prostate and downregulated during carcinogenesis of the prostate gland. *RND3* was further described as a tumor suppressor gene in prostate cancer as its overexpression in DU-145 prostate carcinoma cells induced cell growth arrest and cell death (Table 2). Indeed, ectopic expression of Rnd3 inhibited the expression of cyclin B1 and Cdc2, two proteins essential for the G_2–M transition (32). However, Rnd3 was found overexpressed, together with 14 other genes, in the human prostate adenocarcinoma LNCaP-C4-2 cells, which are associated with a higher metastatic potential when compared to the parental LNCaP cells (40). Yet, the impact of Rnd3 expression on prostate cancer cell invasion has not been evaluated. Altogether, the patient data demonstrated that Rnd3 is downregulated in prostate carcinoma

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Table 2. Consequences of Rnd3 deregulation in cancers

Cancer type	Cell line	Loss or gain of function experiment	Output on cell proliferation	Output on cell invasion	Other output	References
Prostate cancer	DU-145	Gain (overexpression)	Decrease	nd	Induction of apoptosis	(Bektic et al., 2005)
	LNCaP vs. LNCaP C4-2	Increase expression	nd	Increase		(Trojan et al., 2005)
Hepatocellular carcinoma	Hep3B, Huh7	Loss (siRNA)	Decrease	Increase	Epithelial–mesenchymal transition	(Grise et al., 2012)
		Gain (overexpression)		Decrease		
	SMMC-7721, BEL-7402	Loss (shRNA)	No impact	Increase	Membrane blebbing	(Ma et al., 2013)
	PLC/PRF/5	Gain (overexpression)	nd	Decrease		
	MHCC-97L	**Loss (shRNA)**	nd	**Increase**		
	HepG2	Loss (siRNA)	Increase	Increase		(Xia et al., 2013)
Colorectal cancer	T84	Gain (overexpression)	Decrease	nd	Induction of apoptosis	(Poch et al., 2007)
	Lovo	Loss (siRNA)	Increase	nd		(Luo et al., 2012)
	HCT116	Loss (siRNA)	Increase	nd		
	SW620	Loss (siRNA)	nd	Decrease		(Zhou et al., 2013)
		Gain (overexpression)	nd	Increase		
	HCT116	Loss (siRNA)	No impact	nd		(Fu et al., 2014)
Gastric cancer	SGC7901	Gain (overexpression)	nd	nd	Multidrug resistance	(Li et al., 2009)
	AGS, MKN28, KATOIII	Hypoxia-induced upregulation	nd	Increase	Epithelial–mesenchymal transition	(Zhou et al., 2011)
	SGC7901-NM,	Gain (overexpression)	No impact	Increase		(Feng et al., 2013)
	MKN45, MKN28	Gain (overexpression)		Increase		
	SGC7901-M	Loss (siRNA)	No impact	Decrease		
	SGC7901/DDP (resistant to cisplatin)	Loss (siRNA)	nd	nd	Drug sensitivity	(Chang et al., 2014)
Non–small cell lung cancer	H358, H520, A549	Gain (overexpression)	Decrease	nd		(Tang et al., 2014)
Glioma	U87	Gain (overexpression)	Decrease	Increase	Induction of apoptosis	(Poch et al., 2007)
	U251	**Gain (overexpression)**	**Decrease**	nd	Inhibition of Notch signaling	(Liu et al., 2015)
		Loss (shRNA)	**Increase**	nd	Induction of Notch signaling	
	U87	Loss (siRNA)	**Decrease**	Decrease	Induction of apoptosis	(Clarke et al., 2015)
	1321N1, T98G	Gain (overexpression)	Increase	Increase		
Melanoma	A375	Gain (overexpression)	Decrease		Induction of apoptosis	(Poch et al., 2007)
	WM793, WM115	Loss (shRNA)	No impact	Decrease		(Klein and Aplin, 2009)
Sarcoma	Cen3tel	Loss		Increase		(Belgiovine et al., 2010)
Esophageal squamous cell carcinomas	EC1	Loss (siRNA)	Increase	Increase	Reduction of apoptosis	(Zhao et al., 2012)
		Gain (overexpression)	Decrease	Decrease	Induction of apoptosis	
Skin squamous cell carcinoma	SCC4, SCC13, SCC15	Loss (siRNA)	**Increase**	nd	Inhibition of Notch1 signaling, inhibition of cell differentiation	(Zhu et al., 2014)
	A431	Loss (siRNA)	nd	Decrease	Defect in cell–cell contact	(Hidalgo-Carcedo et al., 2011)
	None (DMBA/TPA)	**Loss (Rnd3$^{-/gt}$ mice)**	**Increase**	**nd**	**Transformation**	**(Hernandez-Sanchez et al., 2015)**

NOTE: Bold, *in vivo* data.
Abbreviation: nd, not determined.

but other functional studies are required to address its role in prostate cancer development and progression.

Digestive cancers

Liver cancer. Hepatocellular carcinoma is the main primary malignancy of the liver and one of the most common and aggressive cancers worldwide, causing more than 500,000 deaths annually (41). Work from three independent laboratories, including ours, that included close to 300 cases altogether, highlighted the deregulated expression of Rnd3 in human hepatocellular carcinoma (42–44). Rnd3 was found to be downregulated in the majority of human hepatocellular carcinoma samples at both mRNA and protein levels (Table 1). Interestingly, these studies are complementary as they were done in two different countries (China and France) suggesting that downregulation is not related to patient origin nor to hepatocellular carcinoma etiology.

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Indeed, among the various etiologies linked to hepatocellular carcinoma development, the most prominent is chronic hepatitis B virus infection in southern Asia and hepatitis C virus infection and chronic alcohol consumption in Europe as found in the studied cohorts. In addition, irrespective of the patient cohort and the detection method used, Rnd3 expression was frequently downregulated (66.7%–85% of hepatocellular carcinomas) and by at least twofold in tumor samples. Compared to primary hepatocytes, Rnd3 expression was also found underexpressed in a wide variety of hepatocellular carcinoma cell lines (44). Altogether these data strongly attested of the downregulation of Rnd3 in liver tumors. A consensus about the involvement of Rnd3 in invasion of hepatocellular carcinoma cells and in metastasis development can also be made from the literature. In the French cohort, a correlation between the low level of Rnd3 and the presence of satellite nodules, which are intrahepatic metastases was found (44), and in the Asian cohorts Rnd3 downregulation was associated with shorter disease free survival (42, 43). Thus, low Rnd3 expression may be used as a poor prognosis factor for hepatocellular carcinoma patients. Reproducing Rnd3 downexpression using RNA interference in cultured cells demonstrated that Rnd3 knockdown led to an increase of hepatocellular carcinoma cell invasiveness. This result was robust as different groups showed it in various cell lines (Table 2). However, the mode of invasion was slightly different according to the studied cell type. We found that Rnd3 KD in Hep3B hepatocellular carcinoma cells induced an epithelial–mesenchymal transition with loss of E-cadherin and miR-200b/c, increased expression of ZEB2, and increased activity of Rac/Cdc42 (44). However, Ng and colleagues found that Rnd3 KD in Chinese tumor-derived cell lines activated the RhoA/ROCK pathway favoring an amoeboid mode of migration (42). These studies obtained with different hepatocellular carcinoma cell lines also produced different results in terms of cell growth (Table 2). Indeed, loss of function of Rnd3 led to cell growth inhibition in Hep3B and Huh7 (44), whereas it had no impact in SMMC-7721 and BEL-7402 cell lines (42). Further work is required to better decipher the impact of Rnd3 on tumor hepatocyte proliferation. Thus, whatever its impact on cell-cycle progression on liver tumor cells, data demonstrate that Rnd3 is an antimetastatic gene in hepatocellular carcinoma.

Gastric cancers. Although gastric cancer is the fifth most common malignancy in the world, it is the third leading cause of death (45). Overall incidence rates for gastric cancer have steadily declined over the past 50 years, particularly in developed countries, mainly because of anti-*Helicobacter pylori* therapies. However, resistance to chemotherapy and development of metastases are the major causes of patient mortality.

Two recent studies demonstrated that Rnd3 expression is significantly higher in gastric cancer tissues as compared with normal gastric tissues (46, 47) and even more in metastatic lymph node tissues (ref. 46; Table 1). This upregulation correlated with the differentiation grade and the prognosis of the patient, presenting Rnd3 as a prognosis marker and a metastatic-promoting gene for gastric cancer (46). In cultured gastric cells, Rnd3 expression was also linked to chemotherapy drug resistance. Indeed, the knockdown of Rnd3 enhanced the sensitivity of SGC7901/DDP cells, which are resistant to cisplatin (47), whereas its ectopic expression enhanced the resistance of the parental SGC7901 cells to several kinds of antitumor drugs (48). Few studies further described the possible sources of Rnd3 deregulation in gastric tumor cells (Table 2). The expression of Rnd3 was described to be regulated at the transcriptional level by Hif1alpha in response to hypoxia (49) and at the posttranscriptional level by miR200c (47) in gastric cancer cells. An additional study also demonstrated that its expression might be regulated at the epigenetic level and subject to histone deacetylation (33). The role of Rnd3 in gastric cancer has also been addressed in functional studies. Gain-of-function experiments clearly demonstrated that Rnd3 increases the invasive capacities of various gastric cancer cell lines *in vitro* and *in vivo* (46, 49). The way Rnd3 promotes cell invasion in gastric cancer cells still requires some investigations, but Rnd3 upregulation was shown to induce epithelial–mesenchymal transition in response to hypoxia (49) and to favor invasion through the upregulation of CXCR4, the receptor of the CXCL12 cytokine involved in metastasis development (46). Altogether, in gastric cancers, all data point a protumorigenic role of Rnd3 that could favor metastasis.

Colorectal cancers. Colorectal cancer is a common malignant tumor, where the prognosis is directly correlated with the extent of tumor invasion and metastasis. Studies describing Rnd3 expression in colorectal cancer produced conflicting data. Indeed, two papers using both Chinese cohorts of patients reported opposite results. On one hand, Luo and colleagues (50) demonstrated that *RND3* acts as a tumor suppressor gene in colorectal cancer. In this study, the patient data are mainly based on an immunohistochemistry approach, which is not quantitative. Rnd3 was found to be significantly lower in colorectal cancer tissues ($n = 41$) in comparison to normal tissues ($n = 49$). In the same paper, the quantitative analysis performed by qRT-PCR on 19 colorectal cancer revealed an underexpression of Rnd3 in less than one third of the tumors (Table 1). But, interestingly, the cause of this alteration of expression was suspected to be posttranslational as Rnd3 expression correlated negatively with miR17 level found to be elevated in colorectal cancer. *In vitro*, miR17 indeed targeted Rnd3 3′UTR and preliminary data using one siRNA show that Rnd3 silencing reversed the cell-cycle arrest caused by inhibiting miR17, suggesting a functional link between miR17 and Rnd3 (50). However, in a cohort of 202 paired tissues, Zhou and colleagues found a significantly elevated expression level of Rnd3 in primary colorectal cancer compared with adjacent normal tissues (51). Moreover, Rnd3 expression was significantly correlated with depth of invasion, lymph node metastasis and distant metastasis. This link between Rnd3 expression and invasiveness was further confirmed by transwell assay using SW620 colorectal cancer cell line. Most importantly, this study found that colorectal cancer patients with strong Rnd3 expression had a 6.8-fold higher risk of relapse than those with negative staining. Consequently, disease-free and overall survivals were significantly poorer for patients with Rnd3-positive tumors than those with Rnd3-negative tumors. Thus, in striking contrast with the first study, Zhou and colleagues described Rnd3 expression as an independent prognosis marker for colorectal cancer patients. In both studies, the tissues were analyzed by immunohistochemistry using two different antibodies and protocols, raising the importance of complementary approaches to confirm the deregulation of Rnd3 expression in patient samples (Table 1). Another point is that in Luo and colleagues study, T1+T2 stage represented only 22% of the cases whereas in Zhou and colleagues, it represented about 57% of the cases. In addition, the existence of distinct molecular

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subclasses of colorectal cancer may also explain this discrepancy. The microRNA miR200b, which targets Rnd3 3′UTR was described as overrepresented in TGFßRII-negative colorectal tumors (about 30% of colorectal cancer) when compared to TGFßRII-positive tumors, suggesting that Rnd3 may be downregulated in these tumors (52). Thus, further work is required to clarify the level of Rnd3 expression and its role in colorectal cancer.

Lung cancers

Lung cancer is the most common cancer among men in terms of both incidence and mortality worldwide and among women has the third highest incidence, and is second after breast cancer in mortality. NSCLC is the major histologic subgroup of lung cancer and its 5-year survival depends on the cancer type and stage. Rnd3 mRNA and protein were early shown to be overexpressed in NSCLC, and were correlated with an unfavorable prognostic in patients, suggesting that Rnd3 expression could be used as a prognostic marker in patients with NSCLC (34, 35). In order to translate into clinical application prognostic biomarkers for NSCLC, Jablons and colleagues selected and validated a prognostic model based on the expression of a limited number of genes. They first generated a 4-gene signature that was prognostic of long-term survival in patients with completely resected lung adenocarcinoma (53). This 4-gene signature included *RND3* in addition to *WNT3A*, *ERBB3*, and *LCK*. Later on, the same group extended this signature and developed a qPCR-based 14-gene expression assay (54). This signature was developed in a cohort of 361 patients and further validated in two other large cohorts of 433 and 1,006 patients (54). This practical molecular assay that worked only for nonsquamous NSCLC allowed the identification of patients with high risk of mortality after surgical resection. This robust signature, that predicted survival better than simple staging, was recently patented (US patent US20130059747/European patent EP2726635A1) and developed as a kit for clinical use. This prognostic assay, that remains to be validated in a prospective study, may help clinicians to adapt the chemotherapy to improve outcomes of their patients. Thus, Rnd3 overexpression is linked to bad prognosis and cancer progression for NSCLC patients. However, functional data produced very recently, demonstrated that Rnd3 is downregulated in three NSCLC cell lines, and that a forced expression of Rnd3 in these cells leads to an inhibition of cell proliferation (55). The present paper also underscores the Notch signaling importance for the regulation of cell proliferation by Rnd3 in this tumor context. But, in this latter paper, only one cell line was from lung adenocarcinoma, which is the major subtype of NSCLC. Furthermore, the impact of Rnd3 expression was not evaluated on lung cancer cell invasion. Thus, the evaluation of the impact of Rnd3 deregulation in NSCLC requires deeper investigations.

Brain tumors

Gliomas, arising from glial cells, are the most common intracranial malignant tumors in humans. Gliomas are categorized into grades I–IV based on the degree of malignancy, as determined by histopathologic criteria. Grade IV glioblastoma multiform are highly aggressive neoplasms, which have an elevated invasive capacity, infiltrating diffusely into regions of normal brain. Rnd3 was early described to affect the proliferation of U87 glioblastoma cells (56). Rnd3 overexpression induced a decrease in the phosphorylation of the retinoblastoma protein, Rb and in the expression of cyclin D1 resulting in an inhibition of U87 cell-cycle progression. Rnd3 expression was further shown to induce apoptosis in the same cell line (Table 2). These results suggest a negative role of Rnd3 in glioblastoma development. This is supported by the recent study from Liu and colleagues (57), reporting that the level of *RND3* mRNA and protein are significantly decreased in human glioblastomas in comparison to normal brains. This level is inversely correlated with Notch activity, tumor size and tumor proliferation, and positively correlated with patient survival time (Table 1). In this study, downregulation of Rnd3 is supposed to promote glioblastoma genesis through an enhancement of Notch activity, Rnd3 being described as a repressor of Notch activity by promoting ubiquitination and degradation of Notch transcriptional complex (NICD, CSL, MAML1). However, in contrast with this study, two recent papers demonstrate that Rnd3 is upregulated in glioblastomas when compared either to low-grade glioma (58) or to paracancerous tissues (59). Clarke and colleagues reveal a switch in the activity of Rho-GTPases between low- and high-grade gliomas and demonstrate that Rnd3 appears as a key regulator of tumor proliferation, migration, and invasion. Moreover, the authors found that Rnd3 expression and genomic copy number are predictive of the clinical outcome (58). These opposite findings about the involvement of Rnd3 in glioblastoma may have several roots, such as the origin of the patient cohort, the method used to analyze Rnd3 expression or the tissues used as controls. Indeed, although Liu and colleagues used brain tissues from patients undergoing surgery for intracranial hypertension as control, Clarke and colleagues compared only low- and high-grade glioma in a small cohort of patients. Moreover, the antibodies used to analyze Rnd3 expression either on tissue extracts by Western blot analysis or on tissue slices by immunohistochemistry were different. Thus, based on these different studies, it is difficult to draw a clear conclusion about Rnd3 involvement in glioblastoma genesis and progression. Additional cohorts and studies are required to consolidate the actual alterations of Rnd3 in glioblastoma multiform.

Rnd3 was also described to be up-regulated in the subependymal giant cell astrocytomas (SEGA), a rare low-grade brain tumor often associated with tuberous sclerosis complex (60). This latter disease is characterized by mutations in *TSC1* or *TSC2* genes responsible for mTOR activity resulting in dysregulation of cell growth, abnormal differentiation, and tumorigenesis. Rnd3 was found as one of the genes significantly upregulated in SEGA versus control brain in a microarray analysis. Moreover, this upregulation of Rnd3 was found to be driven by mTOR activity as rapamycin treatment led to a decrease of Rnd3 expression in SEGA cells (60). Thus, Rnd3 represents one of the candidate markers of SEGAs and, in contrary to glioblastomas, its expression is linked to the benign phenotype and relatively low invasiveness of these tumors.

Skin cancer: melanoma

For melanoma, the most serious type of skin cancer, no data about Rnd3 expression in human samples are available. However, several papers reported functional data about the involvement of Rnd3 in melanoma using *in vitro* experiments. Research in the past decades has provided us with insights into the molecular mechanisms that drive melanoma tumorigenesis

Published OnlineFirst August 23, 2016; DOI: 10.1158/1541-7786.MCR-16-0164

and cancer progression. Notably mutations of *RAC1* were found recently in about 9% of sun-exposed melanoma, but the most common mutation found in melanoma affects the serine/threonine kinase B-RAF. *B-RAF* mutations, resulting in the activation of the protein and the MEK/ERK1/2 pathway, are found in about 60% of melanomas. A study focused on delineating mutant B-RAF regulated targets involved in malignant traits identified Rnd3 as a candidate (61). Rnd3 was found as a downstream effector of the oncogene B-RAF-mediated reorganization of the actin cytoskeleton. Indeed, oncogenic activation of the B-RAF–MEK signaling pathway induced Rnd3 expression in WM793 melanoma cells (61). PLX4720, which selectively inhibits B-RAF kinase activity, decreased Rnd3 expression using a pathway that required the Forkhead transcription factor FOXD3 (62). Interestingly, Rnd3 overexpression was linked to the acquisition of an invasive melanoma phenotype. *In vitro*, Rnd3 depletion in WM793 melanoma cells reduced the invasive behavior of melanoma tumor spheroids in a ROCK1/2-dependent manner (63). This ability of Rnd3 to regulate melanoma cell motility was also found dependent on PDK1 level in A375 melanoma cells, as PDK1 competed with Rnd3 for binding to ROCK1 (26).

Following treatment, patients generally develop resistance to agents targeting mutant B-RAF. Reproducing this phenomenon *in vitro*, the resistant cell subpopulation was shown to retain the ability to invade in a three-dimensional microenvironment. It appeared that B-RAF inhibition using drugs invokes a switch in the utilization of the Rnd3–RhoA signaling pathway. Downregulation of Rnd3 expression and enhancement of RhoA activity participated in residual melanoma cell invasion following pharmaceutical B-RAF inhibition. Thus, *in vivo* data would be of interest to define if the Rnd3–RhoA antagonistic function that regulates cell invasiveness may represent a new therapeutic strategy to prevent melanoma progression. Regarding cell growth, although Rnd3 silencing in MW793 cells has no impact on cell-cycle progression and tumor cell growth (63), ectopic expression of Rnd3 led to a decrease in BrdU positive cells and to an increase in apoptosis in A375 melanoma cells (56). Besides these numerous data obtained in human-cultured cells and demonstrating a clear role of Rnd3 in melanoma cell invasion, studies relating the level of Rnd3 in melanoma patient samples are still missing.

Squamous cell carcinomas

Squamous epithelial cells compose the most superficial layer of the epithelium. Rnd3 expression was found underexpressed in various types of squamous cell carcinomas (Table 1). Rnd3 mRNA and protein levels were significantly downregulated in esophageal squamous cell carcinoma and cell lines, when compared to normal esophageal epithelial cells (64). Rnd3 low expression was further associated with the un-differentiated state of the tumors and the presence of lymph node metastasis in the patients. Functional studies in esophageal squamous cell carcinoma EC1 cell line demonstrated that downregulation of Rnd3 expression promoted cell proliferation, cell invasion, and cell apoptosis though modulation of the PTEN/PI3K/Akt signaling pathway (64). Rnd3 was also implicated in squamous cell carcinomas that arise in skin epithelia. It was described as a transcriptional target of activated Notch1 function, which is often attenuated through loss-of-function mutations in squamous cell carcinoma cells (65). Consequently, skin squamous cell carcinoma showed a significant suppression of Rnd3 expression when compared to normal control tissues. The suppression of Rnd3 expression resulted in an increased proliferation and the failure to commit to differentiation in keratinocytes, thus demonstrating that Rnd3 expression is key for cell fate decision in epithelial tissues (65). Strong arguments also come from the first cancer-related *in vivo* study using Rnd3 KO mice (66). The authors demonstrate that heterozygous $Rnd3^{+/-}$ mice are more prone to develop skin tumors following the application of the chemical carcinogenesis protocol using dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA). In this context, the decrease in Rnd3 expression favors the development of benign papillomas and their progression to squamous cell carcinoma (66). Altogether these data strongly demonstrate that decreased Rnd3 expression in squamous epithelial cells might favor tumor development. However, the way loss of Rnd3 favors metastasis remains unclear as a functional study in A431 epidermoid squamous carcinoma cells implicated Rnd3 in collective cell migration by limiting actomyosin contractility at cell–cell contacts and favoring collective cell invasion (27). Thus, in this case, loss of Rnd3 led to a defect in cell–cell adhesion and squamous cell carcinoma cell collective invasion.

Overview

Rnd3 has a diverse expression pattern, depending on the cancer types (Table 1 and Fig. 3). Rnd3 expression is decreased in prostate cancer and in hepatocellular and squamous cell carcinomas, whereas its expression is upregulated in brain, gastric, and lung cancers. However, most functional studies demonstrated that these alterations are in favor of a protumorigenic role (Table 2). Thus, depending on the tumor types, Rnd3 behaves either as a tumor suppressor, or as a tumor promoter. This apparent controversy is probably linked to the lineage and/or to the mutational status of the cancer cell. Although *RND3* mRNA expression is ubiquitous in normal cells, its expression level varies among tissues and was early shown to be particularly low in brain, pancreas, thymus, testis, and kidney (11, 14). Besides the transcript, the amount of Rnd3 protein could also be differently regulated according to the tissue of origin. Data on expression levels of the Rnd3 protein in human organs are still missing. In addition to this variability of expression in the healthy human tissues, the variability in tumor types may additionally be due to the mutational status. Indeed, deregulated pathways are specific and multiples in each tumor types. Thus, the sources of Rnd3 deregulation are multiple. As a rather good correlation between mRNA and protein levels is usually found in tumors (35, 44, 64), it is likely that Rnd3 deregulation may arise at a genomic, transcriptional, or posttranscriptional levels. As described herein (Table 1), in various tumors such as in glioblastoma, colorectal, or gastric cancers, the alteration of Rnd3 expression involves miRNA deregulation (47, 50, 59). Rnd3 expression is also induced by a variety of stimuli, including hypoxia, DNA damage, growth factors, and hormones, which may explain its deregulation upon cancer progression. Indeed, expression of Rnd3 is under the dependence of mTOR pathway in astrocytomas (60), Notch1 pathway in squamous cell carcinomas and glioblastoma (57, 65), and Hif1α in gastric cancers (49). Even if activation of the Raf pathway has been shown to induce the expression of Rnd3 in melanocytes (61) and epithelial cells of kidney origin (67), expression of Rnd3 was found differently altered in tumors in

Published OnlineFirst August 23, 2016; DOI: 10.1158/1541-7786.MCR-16-0164

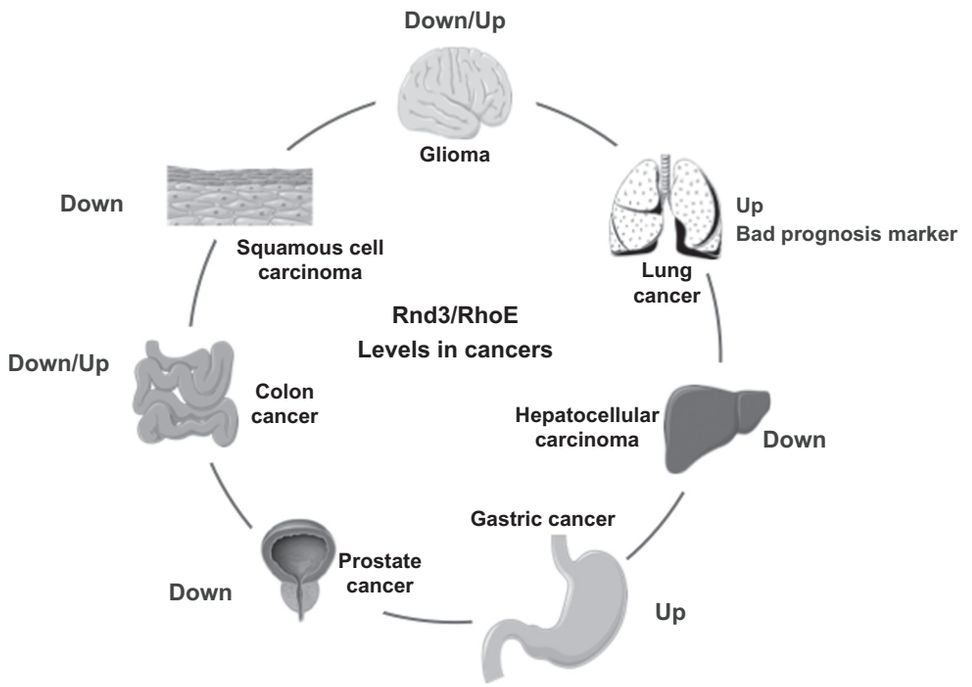

Figure 3.
Rnd3/RhoE expression is differently altered in cancers. Rnd3 expression is either up- or downregulated in human tumors. The figure was produced using Servier Medical Art from www.servier.com (creative commons license, http://creativecommons.org/licenses/by/3.0/legalcode).

which the Ras/Raf/MAP kinase pathway is activated (Table 1), such as colon cancer (51) and skin squamous cell carcinoma (65, 66) with frequent *RAS* mutations, or melanoma (61) that harbor *B-RAF* mutations. Thus, the involvement of Rnd3 in tumorigenesis may be different according to the epithelial cell origin, and the transforming events. Rnd3 was also found as a transcriptional target of p53 in response to DNA damage (68). Given the high frequency of p53 mutations in most cancers, this finding may have relevance for the regulation of Rnd3 expression in such tumor cells. The functions of Rnd3/RhoE in cancer development are complex, which is consistent with the wide range of cellular responses that it modulates (Fig. 2). Certain cancer cells use this signaling pathway to antagonize cell proliferation and migration, whereas others can subvert this pathway to facilitate proliferation, survival, and invasion. It is clear that opposite effects are found upon cancer types, but it is interesting to note that despite its up- or downregulation in tumors, Rnd3 expression is always linked to the promotion of cell invasion and metastasis with the exception of SEGAs. Indeed, reduced expression of Rnd3 in human hepatocellular carcinoma and sarcoma cells increases their invasiveness and metastatic potential, whereas in gastric cancers and glioma, this latter effect is due to the upregulation of Rnd3 (Table 2). The regulation of tumor cell invasion downstream of Rnd3 mainly involved the control of actomyosin contractility through its antagonist role on the RhoA/ROCK pathway (refs. 26, 27, 42, 63, 69; Fig. 2). Interestingly, Rnd3 deregulation was also shown to induce epithelial–mesenchymal transition (EMT) through the upregulation of the EMT factor ZEB2 expression and the downregulation of E-cadherin in tumor liver cells (44). Regarding cell growth, downstream Rnd3 deregulation, various pathways were found to be involved in the regulation of cell-cycle progression and cell proliferation, such as the modulation of the nuclear localization of the DNA licencing protein MCM3 in glioblastoma (58), the regulation of Notch activity in lung cancer (55) and glioblastoma (57), and modulation of cell-cycle proteins (cyclin D1, cyclin B1, Retinoblastoma protein; refs. 30, 32, 56, 70; Fig. 2). Altogether, the impact of Rnd3 expression on cell proliferation is less clear, but the downregulation of Rnd3 expression seems to be associated with an increase of cell growth and reciprocally. This is the case for prostate cancer, squamous cell carcinoma, and glioblastoma. Another important aspect to explain the apparent contradictory results in various tumors is that Rnd3 expression may also be modulated upon the evolution of the tumor. Indeed, we may hypothesize that in some tumors, Rnd3 could be tightly regulated during cancer development. In glioblastoma development, one part of the literature argues for a role of Rnd3 in the switch that transforms proliferative, noninvasive tumors toward nonproliferative and highly invasive tumors. This could be also the case in colorectal cancers, where Rnd3 may be firstly downregulated in order to allow the tumor growth, and then overexpressed to favor invasion and metastasis development.

What's Next? How May the Field Develop?

Rnd3 is clearly a gene of interest to address the mechanisms of carcinogenesis. However, further studies using well-defined cohorts of patients should help to better characterize its level of expression and regulation in various cancers, especially in colorectal cancers and glioblastomas where data are still conflicting. The opposing results obtained in such tumors may also come from the intratumor heterogeneity itself or from the tumor microenvironment. A number of different approaches are currently developed to understand the nature of intratumor heterogeneity or identify the minor populations within the tumor microenvironment. Multiregional biopsies, laser capture microdissection, clonal analysis, or even single-cell sequencing will help to monitor heterogeneity in the context of tumor development or progression (71).

Published OnlineFirst August 23, 2016; DOI: 10.1158/1541-7786.MCR-16-0164

In addition, in light of NSCLC, where Rnd3 is part of a robust 14-genes signature that predicted survival, independent prospective studies are needed to establish the prognostic value of Rnd3 expression for individual patients in other types of cancers. Indeed, given that Rnd3 is involved in invasion of most of tumors, it supports new opportunities for the development of prognostic tools in these cancers.

As an atypical member of the Rho-GTPase family without GTPase activity, the regulation of Rnd3 does not occur through GEFs, GAPs, and GDIs, but rather by a regulation of the transcription/translation and degradation, and by posttranslational modifications. Even if some Rnd3 regulators have been highlighted, there are still much to do to understand the origin of Rnd3 deregulation in cancers. Currently, the large scale sequencing of multiple cancer genomes is carried out, and may help to identify putative *RND3* mutations. The understanding of the transcription factors and the pathways regulating Rnd3 is of particular interest to help developing strategies to either inhibit or favor Rnd3 expression.

Rnd3 plays a dual role in cells; on one hand, it regulates cell proliferation, on the other hand, Rnd3 plays an important role in regulating metastasis and invasion. A more detailed investigation of these two roles of Rnd3 will be necessary to better understand its involvement in the mechanism of carcinogenesis and tumor progression in patients. Indeed, the study of Rnd3 expression and function upon the different steps of cancer development is of interest. Significant advances have been made in modeling cancer genetics in mice. Although xenografts has allowed the rapid *in vivo* assessment of Rnd3 modified cultured tumor cells in immune-compromised mice (42, 65), it is also considered as "animal culture" lacking the tumor microenvironment (72). Thus, mouse models with autochthonous tumors such as genetically engineered models more accurately mimic the pathophysiological and molecular features of human malignancies. In this light, hemizygous $Rnd3^{+/-}$ mice were shown recently to have an increased susceptibility to carcinogen-induced skin tumors with an increase metastatic potential (66). Because of the early postnatal or embryonic death (depending of the studies) of homozygous Rnd3 null mice, development of mice with inducible and/or organ-specific KO is required to address the impact of Rnd3 loss in cancer development in adult mice. These models may overcome the use of cell lines and increase the relevance of the studies as the involvement of Rnd3 in tumor development and progression may then be analyzed either directly or after crossing with cancer-prone mice. These new models may help to better define Rnd3 or the pathways regulated by Rnd3 as therapeutic targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Paysan, L. Piquet, V. Moreau

Writing, review, and/or revision of the manuscript: L. Paysan, L. Piquet, F. Saltel, V. Moreau

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Moreau

Acknowledgments

We are grateful to Dr. Jean Rosenbaum (INSERM UMR1053, Bordeaux, France) for critical reading of the manuscript and support.

Grant Support

L. Paysan was supported by a PhD fellowship from Région Aquitaine. L. Piquet is supported by a PhD fellowship from the SIRIC BRIO. F. Saltel is supported by grants from ANR-13-JJC-JSV1-0005 and "Institut National du Cancer, PL-BIO2015". V. Moreau and F. Saltel are supported by funding from "Equipe Labellisée Ligue Nationale contre le Cancer". V. Moreau is supported by grants from "Association pour la Recherche sur le Cancer" and "Institut National du Cancer, PL-BIO2014."

Received May 9, 2016; revised July 19, 2016; accepted August 10, 2016; published OnlineFirst August 23, 2016.

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Published OnlineFirst August 23, 2016; DOI: 10.1158/1541-7786.MCR-16-0164

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Mol Cancer Res 2016;14:1033-1044. Published OnlineFirst August 23, 2016.