Role of Methylation in Modulating MAPK Signaling. Implications in Melanoma.

Papel de la metilación como modulador de la señalización en MAPK. Implicaciones en melanoma.

Pedro Antonio Andreu Pérez

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PAPEL DE LA METILACIÓN COMO MODULADOR DE LA SEÑALIZACIÓN EN MAPK. IMPLICACIONES EN MELANOMA.

Memoria presentada por

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Para optar al grado de **Doctor en Biología** por la Universidad de Barcelona.

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El Director — El Doctorando

Dr. Juan Ángel Recio Conde — Pedro Antonio Andreu Pérez

Barcelona, Enero de 2012

To my family

ACKNOWLEDGMENTS / AGRADECIMIENTOS

A los laboratorios de Óscar Puig (Helsinki), Juan Ángel Recio (Barcelona) y David Fisher (Boston) por iniciarme, enseñarme y darme la oportunidad de trabajar en ciencia.

A mi abuela, mis padres y mis hermanos por su apoyo constante y dedicación.

CONTENTS

I. INTRODUCTION

1. Cancer Insight

According to the World Health Organization (WHO), cancer is a leading cause of death worldwide, accounting for 7.6 million deaths (around 13% of all deaths) in 2008. Deaths from cancer worldwide are projected to continue to rise to over 11 million in 2030.

It is difficult to define a cancer cell in absolute terms. Cells in multicellular organisms organize and cooperate to promote survival of the organism. In this scenario, cell division, proliferation, and differentiation are tightly controlled to create a balance between normal cell birth and natural cell death (Bishop et al., 2009; McCance KL, 1997). Derangement of these normal homeostatic mechanisms can lead to uncontrolled proliferation or loss of appropriate death, leading to a normal cell taking on a malignant phenotype.

Tumors are usually recognized by the fact that their cells show abnormal growth patterns and are no longer under control. Several evidences indicate that tumorigenesis is a multistep process and that these steps reflect genetic alterations that drive the progressive transformation of normal cells into malignant derivatives. Many types of cancer implicate four to seven stochastic events (Renan, 1993) indicating the multifarious character of cancer.

2. Signal Transduction in Cancer

Cell signaling is the complex system of communication that governs cellular activities and coordinates cell actions. Cell signaling pathways are important for understanding not only cancer progression but also all life phenomena, including regulation of cell growth and death, migration and angiogenesis (Helfrich et al., 2010). In normal cells, different signal transduction molecules accurately control these events. In cancer progression, signal transduction can be irreversible silenced and/or hyperactivated, leading to an impaired signal transduction activity. This loss of control allows cancers to acquire cancer-phenotypes, such as invasion, progression, antiapoptosis and angiogenesis.

2.1 Mitogen Activated Protein Kinases (MAPKs)

Mitogen-Activated Protein Kinases (MAPKs) are one of the best-characterized signal transduction pathways inside the cell. MAPKs are protein Ser/Thr kinases that convert extracellular stimuli into a cellular response. MAPKs are among the most ancient transduction pathways and are widely used through evolution in many cellular processes (Widmann et al., 1999). All eukaryotic cells possess multiple MAPK pathways that coordinate and regulate gene expression, cell division, metabolism, motility, survival, differentiation and apoptosis. Each group of conventional MAPKs is composed of three evolutionary conserved and sequentially acting kinases: A MAPK, a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK). MAPKKKs are protein Ser/Thr

kinases and are often activated through phosphorylation and/or as a result of their interaction with a small GTP-binding protein of the Ras/Rho family in response to extracellular stimuli. MAPKKK activation leads to the phosphorylation and activation of a MAPKK, which then stimulates MAPK activity through dual phosphorylation on Thr and Tyr residues within a conserved Thr-X-Tyr motif located in the activation loop of the kinase domain subdomain VIII. Phosphorylation of these residues is essential for enzymatic activities (Robbins et al., 1993).

ERK1 was the first mammalian MAPK to be cloned and characterized. It was found to be phosphorylated on Tyr and Thr residues in response to growth factors (Ray and Sturgill, 1988) and is expressed to various extents in all tissues (Boulton et al., 1990). ERK pathway regulates cell fate decisions and can be activated by receptor tyrosine kinase (RTK), cytokines and heterotrimeric G-protein-coupled receptors (Wellbrock et al., 2004a). ERK cascade begins when ligands, such as growth factors, bind to their respective receptor tyrosine kinase (RTK), whose dimerization triggers the intrinsic tyrosine-kinase activity of the pathway and activates RAS small G protein, which activates the signaling cascade by phosphorylating a RAF MKKK (c-RAF1, ARAF, BRAF). RAF proteins, in turn, phosphorylate the MEK MKKs (MEK1 and MEK2), which then phosphorylate the ERK MAPKs (Extracellular signal-Regulated Kinases ERK1 and ERK2). Activated ERKs then translocate into the nucleus, where they phosphorylate specific substrates that are involved in the regulation of various cellular responses (Robbins et al., 1994) (see Figure 1). While classical activation of ERK has been considered primarily mitogenic, ERK activation can also regulate differentiation, senescence and survival.

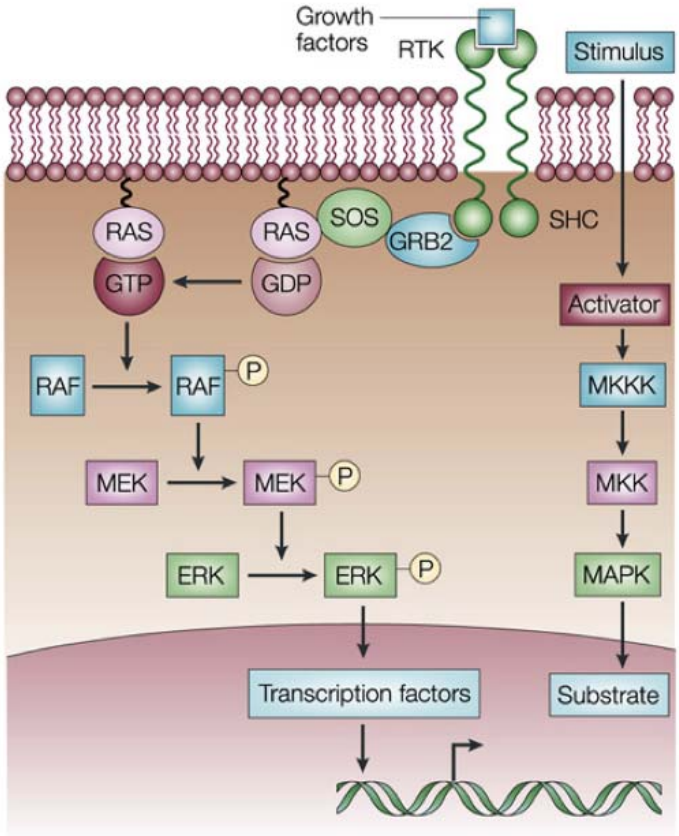

Figure 1: The MAPKs (Mitogen-Activated Protein Kinases) are part of the phospo-relay system that translates a plethora of extracellular signals into diverse cellular responses. After the binding of the growth factors, the receptor tyrosine kinase (RTK) undergoes dimerization that triggers the intrinsic tyrosine-kinase activity. This is followed by autophosphorylation of the receptor that complex with other partners, such as SOS (Son of Sevenless) or GRB2. SOS binds to GDP-RAS that then changes its conformation to GTP-RAS, which is the activator of the signaling cascade. RAS phosphorylates a RAF MKKK (ARAF, BRAF or CRAF). RAF, in turn, phosphorylates the MEK MKKs (MEK1 and MEK2), which then phosphorylate the ERK MAPKs (extracellular signal-regulated kinases ERK1 and ERK2). Activated ERKs then translocate into the nucleus where they phosphorylate specific substrates that are involved in the regulation of various cellular responses (*Adapted from Chin et al., 2003*).

For instance, ERK activated protein kinases can phosphorylate enzymes that regulate cell metabolism, or cytoskeletal proteins that regulate shape and migration (Chin et al., 2006). Into the nucleus, ERK can regulate gene expression by phosphorylating transcription factors (Paumelle et al., 2002) or other protein kinases (Crews et al., 1991).

2.2 MAPKs in Cancer

The RAS-ERK pathway has long been associated with human cancers because *RAS* is mutated to an oncogenic form in about 15% of human cancers (Davies et al., 2002) and ERK is activated in 30% of cancers (Allen et al., 2003).

RAF proteins were discovered 28 years ago by Ulf Rapp (Rapp et al., 1983) and have been since intensely studied. Most of that time researchers have focused on CRAF (also known as Raf-1) as an effector of the RAS proteins. However, reports indicating that BRAF is a human oncogene have highlighted the importance of this family. RAF proteins share three conserved regions: CR1 and CR2 in the N terminus are mainly regulatory, whereas CR3 at C the terminus encompasses the kinase domain. Regulation of RAF proteins is complex and involves many steps, one of those essential steps being RAF proteins recruitment to the plasma membrane by RAS and then the phosphorylation of several amino acids within the activation segment of the kinase domain (Zhang and Guan, 2000). Phosphorylation of a motif called the negative-charged regulatory region (N region) is also

required and it has important implications in the regulation of the RAF isoforms (Mason et al., 1999).

BRAF mutations have been also found in a variety of tumor types including papillary thyroid tumors, colorectal cancers and ovarian cancers (Beddingfield, 2003; Ciampi and Nikiforov, 2005). The majority of the BRAF mutations are activating ones, according to their ability to stimulate ERK signaling *in vivo* (Davies et al., 2002). Many of these residues mutated in BRAF are highly conserved within the RAF family, as they serve key catalytic functions. When mutated in BRAF, they can be activating but they also lower their levels of activity compare to nonconserved residues, explaining the wide range of BRAF mutants' activity. One of those activating mutants is ^{V600E}BRAF: its *in vitro* kinase activity presenting 500-fold greater activity than that of ^{WT}BRAF. In murine cells ^{V600E}BRAF constitutive stimulates ERK signaling, induces cell proliferation and transformation and allows the cells to growth tumors in nude mice (Houben et al., 2004; Ikenoue et al., 2004), indicating that BRAF is an oncogene.

Despite that many of the residues that are mutated in BRAF are conserved among the RAF family and CRAF amplifications (4%) and deletions (2.2%) are strongly associated with tumor progression and overall poorer survival in bladder cancer (Simon et al., 2001), somatic CRAF and ARAF mutations are extremely rare in human cancer. This can be explained by the absence of N region-mediated small lobe stabilization, which is present in BRAF. Therefore, substitutions that reorganize the activation segments in ARAF and CRAF are not sufficient to induce and active conformation, and will not be selected in cancer.

In addition to this, the basal kinase activity of BRAF is higher that that of CRAF or ARAF (Emuss et al., 2005; Pritchard et al., 1995), providing a potential rationale for the frequent mutational activation of BRAF, but not CRAF or ARAF, observed in human tumors. Interestingly, there is a group of BRAF mutants with impaired kinase activity than that of ^{WT}BRAF (Houben et al., 2004; Ikenoue et al., 2004), but they still can stimulate ERK signaling in cells because they activate CRAF, which in turns activate MEK. Indeed, depletion of CRAF in cells expressing these mutants blocks ERK activity (Wan et al., 2004), demonstrating that they can associate with CRAF and transmit the signal regardless their kinase activity, and indicating the dependence of these mutants on CRAF signaling downstream the pathway.

Given the central role of the RAS-ERK signaling pathway in mediating growth and proliferation-promoting signals, it is essential to understand how the different components of the pathway are modulated. For instance, the molecular events required for the activation of the RAF proteins are not fully understood yet: CRAF is the most intensively studied of the RAF isoforms, but the events surrounding its activation remain controversial. Activation of CRAF begins with RAS binding to the RAS-binding domain (RBD) of RAF. As RAS is attached to the inner leaflet of the plasma membrane, this binding recruits CRAF to the plasma membrane, although is not sufficient to trigger CRAF kinase activity (Hancock, 2003). Other signaling molecules, such as 14-3-3 proteins, can also affect this binding. 14-3-3s are scaffold proteins that bind to other proteins in a phosphorylation-dependent manner and they bind to CR2 domain in CRAF when serine 259 is phosphorylated, preventing the binding to

RAS (Light et al., 2002). The presence of four distinct RAS proteins in humans (HRAS, NRAS, KRAS-A, KRAS-B) with different binding affinities for the RBD in CRAF (Weber et al., 2000) add an extra level of complexity to a process that is not fully understood yet. What is clear is that CRAF activation process is highly complex and involves membrane recruitment by RAS, dimerization, conformational changes, binding to other proteins and phosphorylation. Although many of these events seem to be conserved for ARAF and BRAF, there are also crucial differences (Wellbrock et al., 2004a). Another area that needs further study is the role that CRAF plays in mediating the oncogenic activity of the BRAF mutants and how BRAF activates and modulates CRAF. Interestingly, ARAF and CRAF have other functions that appear to be independent of their kinase activity: in many systems CRAF is required for promoting cell survival, but its kinase activity towards MEK seems to be largely unnecessary. Understanding the tight regulatory mechanism of the RAF proteins activation is essential to highlight their role in melanoma and other cancers.

2.3 Protein Arginine Methyl Transferases (PRMTs)

Transmitting a signal from the cell surface to the transcription machinery is a complex process subjected to different kinds of modulating events. Signal transduction pathways, such as MAPKs, undergo posttranslational modifications in response to external stimuli or changes in the environmental conditions. Such modifications alter protein function in very specific ways. Posttranslational modifications such as phosphorylation or acetylation have been extensively studied.

Recently, the importance of other types of proteins modifications have begun to be recognized with methylation standing beside phosphorylation as major modification controlling protein functions: protein arginine methylation is a posttranslational modification involved in a number of cellular processes, including histone modification (Smith and Denu, 2009), structural chromatin remodeling, cell proliferation (Bedford, 2007), DNA repair (Lake and Bedford, 2007), gene transcription regulation and signal transduction (Bedford and Clarke, 2009). Although arginine methylation activity was observed over 40 years ago (Paik and Kim, 1967), only recently the proteins of this enzyme family have been identified (Krause et al., 2007) and the role of arginine methylation as a posttranslational modification fully appreciated as a key process in cellular function.

Arginine is an unique amino acid as its guanidine group contains five potential hydrogen bond donors that are positioned for favorable interactions with biological hydrogen bond acceptors (Bedford and Clarke, 2009). Protein Arginine Methyl Transferases (PRMTs) enzymes remove one methyl group from the donor molecule *S*-adenosyl-L-methionine (AdoMet) and they transfer it to the terminal nitrogen of the guanidine group of an individual arginine residue in the target protein (Gary and Clarke, 1998). There are two guanidine groups (ω) that can be methylated, indeed mono and dimethylation reactions can occur in mammals. Each addition of a methyl group to an arginine residue changes its shape and removes a potential hydrogen bond donor, which might promote the preferential inhibition by methylation of some binding partners (Wolf, 2009). Therefore, modification by

arginine methylation in proteins can modulate their binding capabilities and thus, their physiological functions.

Three different types of methylated arginine can occur in mammalian cell lines. The most common one is omega-N^G,N^G-dimethylarginine (also known as asymmetric dimethylarginine or ADMA) (W.K Paik, 1980). Here two methyl groups are placed on one of the terminal nitrogens of the guanidine group. The other two arginine-methylated derivatives include the symmetric dymethylated derivative (SDMA), where one methyl group is placed on each of the terminal guanidino nitrogens (omega-N^G,N'^G-dimethylarginine), and the monomethylated derivative (MMA) with a single methyl group on the terminal nitrogen atom (omega-N^G-monomethylarginine; MMA) (W.K Paik, 1980). These three derivatives are present on multiple distinct proteins in the cytoplasm and nucleus of mammalian cells (Bedford and Richard, 2005).

PRMTs are classified depending of the type of methylated arginine that they produced: Type I PRMTs (PRMT1, 3, 4, 6 and 8) catalyze the production of ADMA, whereas type II PRMTs (PRMT5 and 7) catalyze the formation of SDMA (Gary and Clarke, 1998). PRMT7 can also monomethylate (catalyzes the formation of MMA) on certain substrates (Miranda et al., 2004), without following SDMA catalysis (Type III PRMTs). PRMT2 is the only Type IV enzymatic PRMT. It has been described only in yeast (Niewmierzycka and Clarke, 1999) and catalyzes the monomethylation of the internal guanidine nitrogen atom (see Figure 2). Methylated arginine residues are often flanked by

one or more glycine residues (Gary and Clarke, 1998), although there are many exceptions to this rule.

2.4 PRMTs in Cancer

Despite the large body of information for the prominent role of PRMTs in transcriptional regulation, their physiological function or their involvement in human disease is still not well understood. The initial studies linking the PRMTs to cancer were merely circumstantial, but new reports are highlighting the mechanism of how deregulated PRMT function might transform cells. For instance, PRMT1 mRNA levels have been reported to be higher in some breast cancer cells than in normal cells (Goulet et al., 2007). Another PRMT, CARM1, shows elevated levels in aggressive breast tumors that also express high levels of the oncogenic coactivator AIB1 (El Messaoudi et al., 2006): CARM1 methylates AIB1 and therefore regulates its activity and stability (Feng et al., 2006).

PRMT5 (a type II methyltransferase) is the best evolutionary conserved PRMT5. It is the human homologue of yeast Skb1 and was identified in a yeast two-hybrid screen as a Jack interacting protein (Pollack et al., 1999). Among the PRMTs, the relevance of PRMT5 in cancer biology has become evident, as there are many studies showing that PRMT5 is involved in the regulation of many signal transduction pathways related to cell death and malignant transformation. For instance, PRMT5 methylates promoter histones H3R8 and H4R3, thereby triggering transcriptional silencing of cell cycle regulatory and

tumor suppressor genes (Pal et al., 2007; Wang et al., 2008). Knocking down of PRMT5 expression causes slow growth whereas PRMT5 overexpression correlates with cellular hyperproliferation. PRMT5 also methylates non-histone proteins, such as p53 tumor suppressor gene (Jansson et al., 2008), thereby modifying p53 activity along with other numerous described posttranscriptional modifications that inhibit or enhance p53 tumor suppressor activity (Chuikov et al., 2004; Huang et al., 2006; Shi et al., 2007). PRMT5 activity has been also related to interleukine-2 (IL-2): In Jurkat T lymphocytes PRMT5 siRNA impairs IL-2 gene expression (Richard et al., 2005), demonstrating that arginine methylation is necessary in T-cells for the recruitment of transcription factors and other components during cytokine gene expression. Recently it has been also reported that PRMT5 accelerates tumor growth by arginine methylation of the tumor suppressor Programmed Cell Dead 4 (PDCD4): this study shows that PRMT5 levels determine long-term survival in PDCD4-upregulated breast tumors (Powers et al., 2011). In addition to all these, the functions of PRMT5 are not fully understood yet. PRMT5 has been reported to have methyltransferase activity towards MPB (Pollack et al., 1999) and Sm proteins (Meister et al., 2001) forming a complex called methylosome (Friesen et al., 2001), and PRMT5 mice homozygous for a null allele display embryonic lethality before somite formation with failure of inner cell mass proliferation (Tee et al., 2010). Therefore, it will be of great interest to determine the functions of PRMT5, to identify the nature and effects of its interaction with other proteins and to determine the biological consequences of protein arginine methylation.

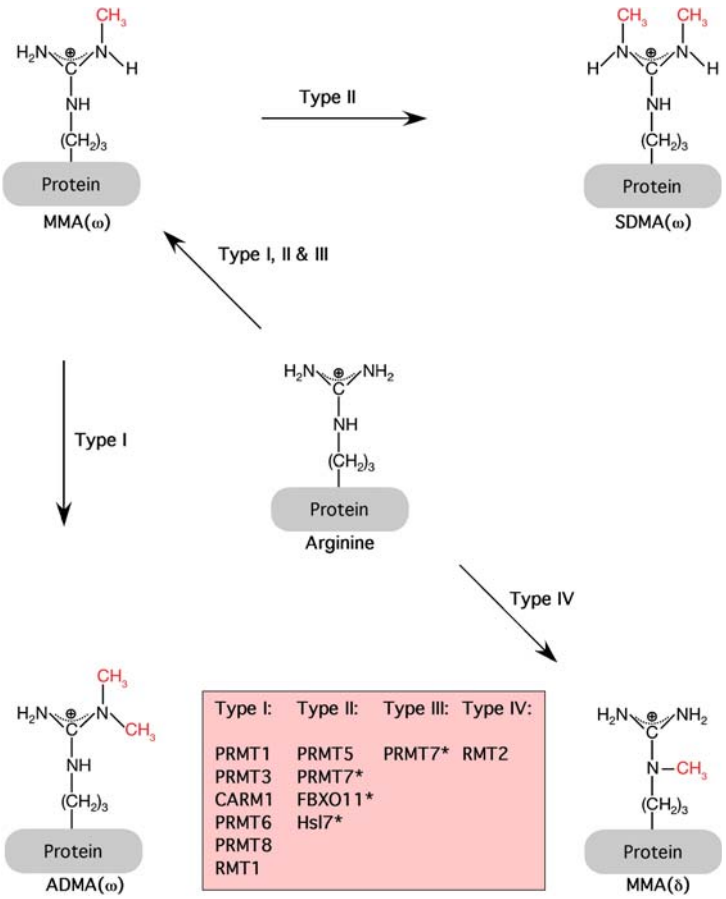

Figure 2: Types of Methylation on Arginine Residues. Types I, II, and III PRMTs generate monomethylarginine (MMAω) on one of the terminal (ω) guanidino nitrogen atoms. These two nitrogen atoms are equivalent. The subsequent generation of asymmetric dimethylarginine (ADMA) is catalyzed by type I enzymes, and the production of symmetric dimethylarginine (SDMA) is catalyzed by type II enzymes. Type III PRMTs only monomethylate. Type IV enzyme activity that catalyzes the monomethylation of the internal guanidino nitrogen atom has only been described in yeast. The type of methylation reactions catalyzed by PRMT7, FBXO11, and Hsl7 are still being established and are, thus, marked with an asterisk (*Adapted from Bedford and Clarke, 2009*).

3. Melanoma

Melanoma is the most lethal form of skin cancer: a 4mm tumor thickness presents a high risk of metastasis, and diagnosis of metastatic melanoma carries a median survival of 6-9 months. Its incidence is influenced by factors such as pigmentation of the population and geographical parameters (altitude and latitude), suggesting a strong correlation between ultraviolet (UV) light exposure and melanoma risk (Armstrong and Kricker, 2001). The current increase in melanoma incidence can be explained, in part, by altered patterns of sun exposure related to increased popularity of sun-tanning, and the increase in travel or migration of fair-skinned individuals to sun-intense regions (Gilchrest et al., 1999).

The first information available about melanoma dates from 1820, when William Norris reported a predisposition to melanoma in individuals with light-colored hair and pale complexions (Norris, 1820). Only two centuries later we are beginning to understand the genetic and environmental factors that drive melanoma progression and development: melanomas often arise from the malignant transformation of pigment-producing melanocytes that reside at the epidermal-dermal junction of the human skin (Kabbarah and Chin, 2006), although they can also derive from noncutaneous melanocytes such as those in the choroidal layer of the eye, mucosal surfaces or the meninges. Clinically, four subtypes of melanoma exist: (1) Lentigo maligna is associated with chronic sun-exposed areas of the body, such as the face. (2) Acral lentiginous melanoma is found on non-sun-exposed regions, such as the palms, nail beds and soles of the feet. (3)

Nodular melanoma is a raised nodule that may or may not have an associated superficial spreading component. The most common is (4) superficial spreading melanoma, which generally occurs on areas of the body with intermittent sun exposure, such as the trunk and proximal extremities (Gray-Schopfer et al., 2007). Clinical identification of primary melanomas includes measurement of thickness, presence of ulceration, mitotic rate, tumor spread to draining lymph nodes and evidence of metastasis.

Melanoma progression can begin with the development of either dysplastic or benign nevi, which can then progress to the radial growth phase: at this stage cells are still dependent on growth factors and they are not anchorage-independent or tumorigenic. In this phase the growth expands laterally but remains localized to the epidermis. The primary lesion can also progress to the vertical growth phase, characterized by invasion of the dermis, subcutaneous tissue and upper dermis. In this phase, cells are no longer dependent on growth factors, are anchorage independent and presage metastasis (Ghosh and Chin, 2009).

Several critical biological questions arise from the study of melanoma: (1) What are the genetic or environmental factors contributing or modulating the development and maintenance of melanoma? (2) Are there biological markers in early melanoma lesions that can predict the risk or metastasis? (3) Which genetic alterations are responsible for development and progression of melanoma? (4) What biological or molecular pathways are melanoma maintenance-

essential? (5) Are any of those pathways targetable for therapeutic intervention in humans?

At present day, early detection and prevention are the only effective tools against melanoma. However, with the advent of new technologies and model systems our understanding of the melanoma insights are expanding, along with the potential development of drugs and biomarkers for the disease monitoring, which might impact positively in the detection and treatment of melanoma. In this scenario, genomic approaches are highlighting the importance and relevance of genes that can be validated in mouse models.

3.1 MAPKs in Melanoma

ERK protein kinase is hyperactivated in 90% of human melanomas by growth factors and genetic alterations of upstream effectors: *RAS* and *BRAF* genes are mutated in 30% and 70% of human melanomas respectively (Davies et al., 2002). The most common substitutions are gain-of-function mutations that lead to their permanent activation and therefore promotion of proliferation, survival, invasion and angiogenesis in melanoma (Gray-Schopfer et al., 2005). Consistent with the involvement of MAPKs signaling in tumorigenesis, activating mutations in the RAS family of proto-oncogenes (*HRAS*, *NRAS* and *KRAS*) have been detected in melanoma, ranging from 10% to 15% incidence (Chin et al., 1998). The RAS proto-oncogenes have the ability to induced and/or maintain the transformed state when they are over expressed or altered by

mutations or gene arrangements. *NRAS* has significant association with melanoma progression: several studies have reported *NRAS* activating mutations in 56% of congenital nevi (Papp et al., 1999), 33% of primary melanomas and 26% of metastatic melanoma samples (Demunter et al., 2001). In addition to this, *NRAS* mutations have also been correlated with nodular lesions and sun exposure (Jafari et al., 1995; van Elsas et al., 1996). On the genomic level, it has been reported that chromosome 11p, where *HRAS* is encoded, is occasionally amplified leading to an amplified *HRAS* allele that possesses oncogenic point mutations in Spitz nevi (Bastian et al., 2000). Introduction of *HRAS* can also transform normal human melanocytes, although this event occurs rarely (Greenhalgh et al., 1994). The different *HRAS* and *NRAS* mutation patterns could indicate the existence of two different evolutionary paths to melanoma: whereas activated *HRAS*, together with inactivating mutations in *Ink4a*, *Arf* and/or *p53* promotes development of non-metastatic melanoma, expression of dominant active *NRAS* in *Ink4*-deficien mice drives metastasizing melanocytic tumors (Chin et al., 1997; Demunter et al., 2001).

BRAF is a potent activator of ERK, and BRAF mutations are the most prevalent genetic event in human melanoma, which incidence range from 27% to 70% (Maldonado et al., 2003). 50% of all melanoma cases present activating mutations in BRAF (Davies et al., 2002). More that 95% of these mutations affect a valine residue at the 600 amino acid position, resulting in a constitutively active kinase that hyperstimulates MEK kinase. The introduction of this single altered gene is sufficient to transform *p16* null melanocytes (Wellbrock et al., 2004b), indicating that BRAF is an oncogene in melanocytes. The list of

oncogenes and tumor suppressors that contribute to melanoma pathophysiology may not be completed, neither it is the list of regulatory process and/or changes that drive the MAPKs behavior in melanoma. Because of this, studying the mechanisms that govern MAPK signaling in melanoma is essential to understand the basics of melanoma progression and to develop new-targeted therapies.

3.2 Genetics of Melanoma: the importance of 9p21 locus

There are several physical features, like blue eyes, red hair, inability to tan, etc. that correlate with an increased risk of melanoma (Gilchrest et al., 1999). In the same way, the presence of multiple pigmented lesions like freckles or moles is also associated with increased risk of melanoma (Gandini et al., 2005). However, one of the most significant risk factors for melanoma occurs in individuals with a family history in melanoma. Linkage-analysis studies led to the identification of the chromosomal region 9p21 as directly related with melanoma susceptibility (Hussussian et al., 1994). 9p21 chromosome is a cancer hot spot identified when homozygous deletions centered on *CDKN2A* gene were observed in cancer cell lines of many different types (Nobori et al., 1994). CDKN2A locus has been designated as the familial-melanoma locus because mutations in this gene correlate with high melanoma susceptibility in people with familial melanoma (Hussussian et al., 1994). In order to confirm the importance of CDKN2A loss in 9p21-mediated melanoma, exons 2 and 3 were deleted by a gene targeting approach in mouse germline. These mice developed fibrosarcomas and lymphomas (Serrano et al., 1996) and

became highly prone to cutaneous melanoma with short latency when combined with an activated *HRAS* mutation in their melanocytes (Chin et al., 1997).

The CDKN2A locus presents a high complexity level of genomic organization as it encodes for two distinct tumor suppressor proteins: INK4A (also known as $P16^{INK4}$) and ARF ($p14^{ARF}$ in humans and $p19^{ARF}$ in mice). *CDKN2A* gene contains two upstream exons, 1α and 1β, that are driven by different promoters. This results in alternatives transcripts that share common downstream exons 2 and 3. Different reading frames in exon 2 give rise to two different products: the transcript initiated in exon 1α encodes INK4A (Serrano et al., 1993) whereas the second transcript initiated at exon 1β encodes ARF (Quelle et al., 1995), (see Figure 1a). Both proteins share no amino acid homology yet each of them posse strong anticancer activities: loss of INK4A (Inhibitor of cyclin-dependent Kinase 4) function promotes retinoblastoma (RB) protein inactivation through hyperphosphorylation (see Figure 1b), resulting in unconstrained cell cycle progression (Serrano et al., 1993). Around 25%-40% of familial melanomas have mutations in the INK4A coding region (Tsao et al., 2000). Alterations in *CDK4* and *RB* have also been associated with melanoma in humans, albeit rarely (Wolfel et al., 1995). On the other hand, ARF (the Alternative Reading Frame product of the CDKN2A locus) functions as a potent growth suppressor that stabilizes and enhances p53 levels by blocking MDM2-mediated p53 ubiquitination and degradation (Kamijo et al., 1998; Pomerantz et al., 1998; Zhang et al., 1998); therefore loss of ARF inactivates p53 (see Figure 1c), a must step in virtually all tumor cells.

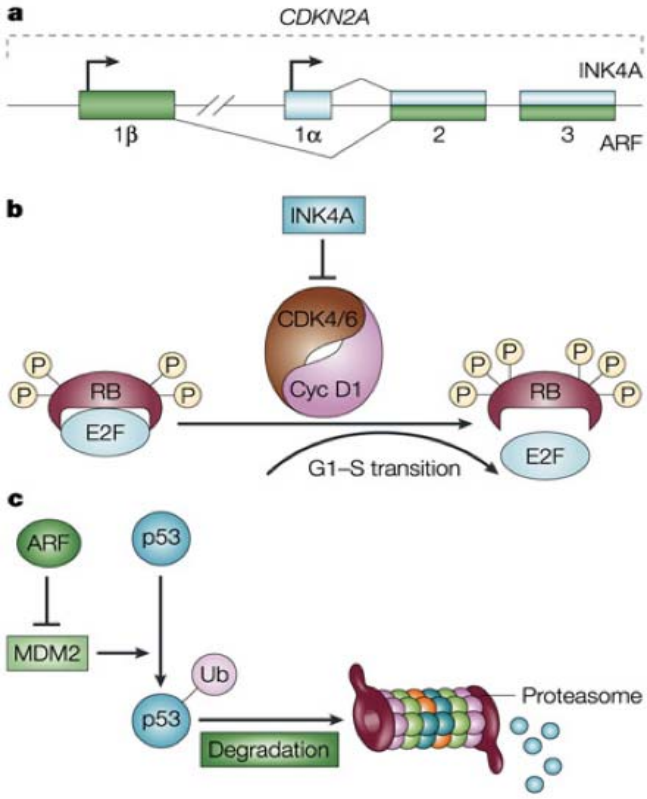

Figure 3: The 9p21 locus presents an unusual degree of genomic organization. a) The *CDKN2A* gene contains two upstream exons, 1α and 1β, driven by separate promoters, which result in alternative transcripts that share common downstream exons 2 and 3. The transcript initiated from the proximal promoter (1α) encodes INK4A. b) By inhibiting CDK4/6–cyclin-D-mediated hyperphosphorylation of RB, INK4A ensures that RB remains in complex with the E2F transcription factor. These RB–E2F complexes recruit histone deacetylase to promote and repress transcription of target genes, leading to G1 arrest. In the absence INK4A, CDK4/6–cyclin-D phosphorylates RB, which results in the release of E2F that activates genes that are necessary for progression into S phase. c) The second transcript initiated from the upstream exon 1β, encodes ARF. ARF functions as a potent growth suppressor that stabilizes and enhances p53 levels by blocking MDM2-mediated p53 ubiquitylation and degradation. (*Adapted from Chin et al., 2003*).

Several genetic studies have unveiled the importance of *CDKN2A* in 9p21-mediated melanoma suppression, but despite its important role, mutations in *CDKN2A* only account for a proportion of melanomas, indicating that there must be additional relevant genes playing a role in melanoma maintenance and development. One of the interesting genes that may play a role in melanoma is *MTAP*. The MTAP locus lies on the 9p21 chromosome, close to the CDKN2A locus that encodes INK4 and ARF proteins. The first review on tumors lacking MTAP was published in 1988 (Carson et al., 1988) and since then, new information from many sources on the incidence of MTAP-deficiency have been reported: MTAP locus is frequently deleted in human melanoma (Chin et al., 2006) and both *MTAP* and *CDKN2A* genes are often homozigously co-deleted and associated with melanoma risk (Bishop et al., 2009). In addition to this, selective *MTAP* deficiency without co-deletion with CDKN2A locus has also been reported (Behrmann et al., 2003) and genomic wide-association studies have identified the loss of *MTAP* as a melanoma risk factor associated with the development of cutaneous nevi (Bishop et al., 2009; Falchi et al., 2009)

3.3 Methylthiodenosine Phosphorylase (MTAP) and Melanoma

In normal cells, MTAP catalyzes the phosphorylation and cleavage of methylthioadenosine (MTA), generated during the biosynthesis of spermidine and spermine polyamines, in order to produce adenine and 5-methylthioribose-1-phosphate (Avila et al., 2004). This latter compound is metabolized to methionine and adenine is converted to

AMP. Cells lacking MTAP are unable to salvage methionine and adenine from endogenous MTA, which is not cleaved and accumulates.

MTA is a potent inhibitor of methyltransferases and it has been shown that concentrations in the micro molar (μM) range are able to interfere with cell proliferation, invasiveness, tumor development and control of the apoptosis (Ansorena et al., 2002; Chen et al., 2007; Pascale et al., 2002). It has also been published that MTA presents anti-proliferative inhibitory properties in liver and colon cancer cells (Ansorena et al., 2002; Li et al., 2009), but strikingly there are no MTA data referring to melanoma. As *MTAP* loss, which is frequently deleted in human melanoma, correlates with an increase in the intra and extracellular MTA levels (Kirovski et al., 2011; Stevens et al., 2009), a number of questions about the effects of MTA remain unclear: (1) What are the consequences of the *MTAP* loss and MTA accumulation within the cells (2) Does MTA present any therapeutic potential for melanoma treatment?

II. OBJECTIVES

1. Investigate the effects of the deregulation of MTA amounts on melanoma cells.

2. Identification of the molecular mechanism/s related to the MTA effect.

3. Determine the therapeutic potential of MTA for melanoma treatment.

III. DIRECTOR´S REPORT ON THE JOURNAL´S IMPACT FACTOR

III. INFORME DEL DIRECTOR DEL FACTOR DE IMPACTO DE LOS ARTÍCULOS PUBLICADOS

El trabajo de investigación realizado por Pedro Antonio Andreu Pérez en relación a las consecuencias moleculares de la desregulación de los niveles de metiltioadenosina (MTA) así como el descubrimiento del mecanismo bioquímico implicado que afecta a la vía de señalización celular de RAS y su posible explotación terapéutica en el tratamiento del melanoma, ha sido publicado en revistas internacionales *peer reviewed* indexadas en el ISI.

1.- Methylthioadenosine (MTA) inhibits melanoma cell proliferation and *in vivo* tumor growth.

Pedro Andreu-Pérez, Javier Hernandez-Losa, Teresa Moliné, Rosa Gil, Judit Grueso, Anna Pujol, Javier Cortés, Matias A Avila and Juan A Recio

BMC Cancer 2010, **10**:265 doi:10.1186/1471-2407-10-265

BMC Cancer is an "*open access journal*" con un factor de impacto de 3.15. Este artículo es un artículo catalogado como "**highly accessed article**" por la revista. El número de visitas recibidas hasta el 9 Enero 2012 fueron:

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2.- Protein Arginine Methyltransferase 5 Regulates ERK1/2 Signal Transduction Amplitude and Cell Fate Through CRAF.

Pedro Andreu-Pérez, Rosaura Esteve-Puig, Carlos de Torre-Minguela, Marta López-Fauqued, Joan Josep Bech-Serra, Stephan Tenbaum, Elena R. García-Trevijano, Francesc Canals, Glenn Merlino, Matías A. Ávila, and Juan A. Recio.

Sci. Signal. **4**, ra58 (2011).

Science Signaling es probablemente la revista más prestigiosa dentro del campo de la señalización celular. Este artículo recibió los siguientes comentarios :

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IV. DISCUSSION

In many melanoma cells the loss of MTAP locus is a frequent homozygous deletion (Bishop et al., 2009) that correlates with an increase in MTA levels (as MTAP metabolizes and controls the levels of MTA within the cell). Several studies have shown that elevated concentrations of MTA, a potent inhibitor of methyltransferase activity, can interfere with cell proliferation, tumor development, invasiveness and control of apoptosis (Ansorena et al., 2002; Chen et al., 2007; Pascale et al., 2002). In addition to this, MTA inhibits mitogen-induced proliferative response in liver and colon cancer cells (Ansorena et al., 2002; Li et al., 2009) and has proapoptotic effects in liver cancer cells (Yang et al., 2004). Although the loss of *MTAP* expression in malignant melanoma results in higher invasive potential (Behrmann et al., 2003), there is no data regarding how the loss of *MTAP* and enhanced MTA accumulation might affect the signaling pathways that govern the signal transduction in melanoma. Because of this we decided to study the effects of the addition of MTA in terms of cell signaling, and we found a new regulatory mechanism by which methylation reactions regulate the kinase activity of the main signaling cascade in melanoma, the RAS-ERK pathway, modifying the biological response to a given stimuli. We demonstrate for the first time that protein methylation is a posttranslational modification capable of modulating RAS-ERK pathway activity in response to growth factors. We identify PRMT5 as the arginine methyltransferase involved in the methylation process and we also find a specific PRMT5 GRG motif, which is conserved through evolution in the RAF proteins: methylation at the arginine residue within this motif is controlling RAF proteins stability and kinase activity, thereby modifying the cellular response dependent on the RAS-ERK pathway. We show that inhibition of methylation at this residue can

switch the growth factor-elicited response from proliferation to differentiation in PC12 cell line, highlighting the central role of methylation into modulating the activity of the RAS-ERK pathway and controlling the biological output in response to growth factors.

Our initial observations showed that the addition of the methylation inhibitor 5´-methylthioadenosine (MTA) in the μM range increased the phosphorylation level of ERK protein in mouse melanoma cells. MTA-exerted effect was only present when the RAS-ERK signaling pathway was activated upon growth factor treatment. Interestingly, this effect was ligand dependent and growth factor specific. Furthermore, we found that the enhanced ERK phosphorylation mediated by MTA is a highly specific pathway process since we did not detect any alteration in the PI3K pathway signaling upon growth factor treatment in the presence of MTA.

MTA is a potent inhibitor of methyltransferases suggesting that the process involved in the enhanced ERK phosphorylation was mediated by the inhibition of protein methylation reactions. We confirmed this hypothesis using two well-known protein methylation inhibitors: 3-deaza-adenosine (Deaza) and cycloleucine (c-leucine) did enhanced ERK phosphorylation upon growth factor triggering, indicating that the increased signal amplitude of ERK in response to growth factors in the presence of MTA was due to the inhibition of methylation reactions. Signal transduction occurs through cascades of activation and inactivation, such as the MAPK pathways, that are often composed of kinase and phosphatase pairs. Both kinases and phosphatases exert control in the signal transduction process: in the

RAS-ERK pathway kinases control signal amplitude whereas phosphatases tend to control both amplitude and duration of that signal (Hornberg et al., 2005). Our results showed that the modulation of ERK amplitude by the addition of MTA took only two minutes after growth factor triggering, suggesting that this mechanism was likely affecting kinases rather that phosphatases, thereby indicating the cytoplasmic nature of the MTA-mediated process.

Protein methylation is an important posttranslational modification affecting to protein function. There are two major groups of protein methylation: protein carboxyl methylation and protein nitrogen groups methylation, which includes arginine and lysine methylation (Grillo and Colombatto, 2005). It has been reported a role of protein carboxyl methylation in signal transduction pathways (Philips et al., 1993; Volker et al., 1991) and carboxyl methylation, along with other modifications, in the C terminal CaaX motif of RAS appears as a posttranslational modification necessary for RAS translocation to the membrane and subsequent activation of the pathway (Chiu et al., 2004). Despite the importance of the carboxyl methylation in the RAS activation process, our results showed no alterations in RAS translocation to the membrane upon MTA treatment, suggesting that the protein methyltransferase involved in the process is not directly connected to RAS proteins. The other major group of protein methylation, amino methylation, can take place in lysine or arginine residues. While methylation in lysine residues has been typically associated to histone proteins (Zhang et al., 2012) and is mainly confined to the nucleus, methylation of arginine residues appears to occur throughout the cell (Bedford and Richard, 2005). The

modification of arginine chain side guanidine group is one the most extensive protein methylation modification in mammalian cells (W.K Paik, 1980). There is a well-conserved family of protein arginine methyl transferases (PRMTs) ranging from 1 to 9. It does not seem to be redundancy among them and the diversity of these enzymes is enhanced by alternative splicing reactions that lead to amino acid sequence variants (Goulet et al., 2007). PRMT1 was the first mammalian PRMT identified as a single gene product and has very wide substrate specificity, being responsible for 85% of total methylation reactions in the cell (Tang et al., 2000). Despite the importance and ubiquitous presence of PRMT1, our results have genetically and biochemically discarded PRMT1 and identified PRMT5 as the enzyme involved in the modulation of ERK phosphorylation. PRMT5 is the most highly evolutionary conserved methyltransferase and the first type II methyltransferase identified that can result in the formation of sDMA residues (Branscombe et al., 2001). In contrast to PRMT1, which is known to be mainly found in the nucleus, human PRMT5 is localized in the cytoplasm forming part of the methylosome complex, where proteins are methylated symmetrically to be able to interact properly (Friesen et al., 2001; Meister and Fischer, 2002). PRMT5 dimethylates a number of proteins affecting their function: it methylates Myelin Basic Protein (MBP) (Eylar, 1972), it is required for p53 expression and induction of p53 targets (Scoumanne et al., 2009). PRMT5 also binds to death receptor 4 (DR4) (Tanaka et al., 2009) and modulates EGFR-activity (Hsu et al., 2011). Our results showed that PRMT5 was able to limit ERK phosphorylation in response to growth factors, suggesting that methylation may facilitate the appropriate cellular response to a specific stimulus. Our data also showed that MTA

neither changed the phosphorylation status of c-Met receptor nor affected the amount of active RAS-GTP upon growth factor stimulation, dismissing both RAS and c-Met as probable targets of the methylation process. We also found that MTA ability to induce ERK phosphorylation was impaired in $BRAF^{V600E}$ mutant cells (which signal independently of RAS), but remained intact in $NRAS^{Q61L}$ mutants (which signal through CRAF) (Dumaz et al., 2006), suggesting that this mechanism was RAS activation-dependent. Furthermore, we showed that PRMT5 binds *in vitro* to active recombinant CRAF and HGF-dependent ERK MTA-enhanced phosphorylation was limited in cells where *CRAF* was knocked down, supporting the requirement for RAS to engage PRMT5 in the pathway signaling machinery and pointing out at CRAF as the probably target of the methylation process exerted by PRMT5. In addition to this we also found that BRAF and CRAF proteins were methylated *in vivo* upon growth factor triggering. Strikingly, the methylation degree was different depending on the growth factor, indicating that PRMT5 catalytic activity was differentially modulated in response to different growth factors. In agreement with this, *in vitro* methylation experiments showed that a non-active form of recombinant CRAF could not be methylated by PRMT5, supporting the RAS pathway-dependence activation by growth factors of this mechanism. This data suggest that in addition to phosphorylation, protein methylation activity is regulated in signal transduction upon growth factor triggering.

Symmetric dimethylation of arginine residues in many nucleic-acid-binding proteins takes place in their glycine-rich and arginine-rich motifs (Meister et al., 2001), indicating that the presence of an internal

-Gly-Arg-Gly- (-GRG-) sequence is potentially methylatable by a methyltransferase. We looked for a GRG sequence within the RAF proteins and we found a GRG motif highly conserved in all the RAF isoforms along evolution. To investigate the sequence requirement for methylation of these proteins we designed synthetic oligopeptides containing the CRAF GRG and a mutant form where the central arginine was substituted by a lysine (GKG): our results indicated that PRMT5 *in vitro* methylated the arginine within the GRG motif whereas no methyltransferase activity was observed in the assays with the mutant peptide. The identity of the methylated residue was confirmed by *in vivo* methylation experiments, where $CRAF^{R563K}$ mutant was not methylated in response to growth factors. Furthermore, PRMT5 depletion by siRNA abolished CRAF methylation *in vivo*, confirming the role of PRMT5 in the methylation-mediated process. Together this data presents methylation as a new modulatory mechanism capable of regulating the activity of the RAS pathway in response to growth factor. Furthermore, we show for very first time that a methyltransferase process can modulate the kinase activity of MAPK thereby regulating the signal output and the cellular response.

In agreement with PRMT5´s ability in limiting ERK output in RAS pathway, we observed that depletion of PRMT5 or conservative substitution of arginine 563 for a lysine increased CRAF stability (we also observed this result in BRAF), suggesting that methylation at this arginine residue decreases the stability of the RAF proteins targeting them to the proteosome. In agreement with this, we showed that inhibition of methylation reactions not only increased the kinase activity of CRAF, but it also enhanced the phosphorylation of CRAF

serine 621, which have been shown to be required to prevent proteosome-mediated CRAF degradation (Noble et al., 2008). Interestingly, our results showed that the kinase activity of CRAF or BRAF mutant forms unable of being methylated ($CRAF^{R563K}$ and $BRAF^{R671K}$) was also increased in response to growth factors. This could be explained in part because although methylation does not change the charge of the arginine residue, the addition of methyl groups occupies hydrogens that might be involved in the formation of hydrogen bonds. Therefore, inhibition of methylation may enhance the formation of BRAF-CRAF heterodimers with increased kinase activity. It is also known that active RAS induces heterodimerization of BRAF and CRAF therefore increasing the total activity of the pathway (Rushworth et al., 2006). We can therefore hypothesize that inhibition of methylation process by MTA or PRMT5 siRNA or the use of non-methylatable mutants may enhance the formation of BRAF-CRAF heterodimers and therefore increase their kinase activity.

Cellular decisions with respect to division, differentiation and apoptosis involve signal transduction pathways. The complexity of this networks is elevated because of their large number of interacting constituents, the number of reactions involved, feedbacks and crosstalks, and the fact that the kinetics of interaction matter (Huang and Ferrell, 1996). A central feature of signal transduction downstream of both receptor and oncogenic tyrosine kinase consist in the RAS-dependent activation of RAF, MEK and ERK (MAPKs). The ERK pathway can be activated by different growth factors thereby eliciting different biological responses (Marshall, 1995; Pearson et al., 2001). Although during the last decade the knowledge of the mechanisms involved in

growth factor signaling has been almost overwhelming, the control and transfer of information through signaling networks is not fully understood yet: a subtle difference in the input signals or modifications in the pathway´s components may result in differential response patterns and eventually, in alterations in gene expression by signal regulated transcription factors. In this scenario, the RAS-RAF-ERK signaling cascade appears to play a central role in cell behavior, because depending of the cellular context this pathway can control either proliferation or differentiation (Marshall, 1995). ERK pathway activity is regulated by a large number of posttranscriptional modifications such as phosphorylation or acetylation, but recently other types of protein modifications have taken center stage. Thus, the biological response triggered by a growth factor regarding to the activation of a particular protein kinase can be explained by its kinase phosphorylation profile. In this matter, as ERK activation dynamics are controlled by the configuration of the network in which it is embedded, where the connections with the MAPK signaling module can adopt different configurations depending of the stimulation context (growth factors) and the posttranslational modifications that affect the activity of MAPKs (Santos et al., 2007), we decided to test the hypothesis that CRAF arginine methylation is a key posttranslational modification in growth factor-elicited responses. In order to address that question we used PC12 cells, a model of neural differentiation (Greene and Tischler, 1976). In this model both EGF and NGF growth factors activate the RAS pathway presenting different ERK activation profile. The distinct ERK activation dynamics result in differential gene expression and therefore in a different cellular response dependent on the ERK exhibited activity: on NGF stimulation ERK shows a sustained activation and the

cells do proliferate. EGF stimulation induces ERK transient activation and PC12 cells undergo proliferation (Santos et al., 2007). Our research showed that EGF in combination with the methyltransferase inhibitor MTA completely mimic NGF-induced differentiating response: PC12 cells treated with EGF and MTA differentiated in a manner almost undistinguishable from that of cells treated with NGF, whereas MTA alone or EGF treatment did not induced the differentiation process. Besides, we also found that PC12 cells transfected with a catalytically inactive PRMT5 mutant (PRMT5ΔGAGRG) or with $CRAF^{R563K}$ mutant underwent differentiation in response to EGF. In agreement with this, our results showed that NGF induced less methylation of BRAF than EGF. On the other hand, EGF stimulation in combination with *PRMT5* knock down induced a methylation level similar to that of NGF. This differences in the methylation levels are consistent with the differences in ERK phosphorylation observed upon MTA treatment after activating the pathway with different growth factors (NGF vs. EGF) and explain why methylation can switch the cellular response elicited by a certain growth factor.

When a growth factor activates a MAPK pathway it not only initiates the signal that activate the kinases (thereby controlling signal amplitude) but activates the counterpart mechanisms (phosphatases) to deactivate that signal (thereby affecting signal duration). In our model, absence of PRMT5 in PC12 correlates with an increase in ERK phosphorylation in response to EGF that overtakes the phosphatase activity triggered by the growth factor, resulting in a signal long enough to reach the threshold required to elicit the neuronal differentiation in PC12. Therefore, our research highlights and additional control

mechanism that can explain the differential effect of MAPKs activation observed in PC12. In this scenario, methylation regulates stability of the RAF proteins and modulates the signal output of the pathway, thereby switching the cellular response from differentiation to proliferation.

Our data demonstrate that protein arginine methylation limits MAPK signal transduction in response to growth factor. We show for the first time that PRMT5 (a protein arginine methyltransferase) methylates RAF proteins thereby modulating their stability and kinase activity, limiting their signaling downstream the pathway. This new mechanism shows how RAF proteins are kept in check controlling the activation profile of ERK and consequently the cellular fate in response to growth factors.

As our data presented protein methylation as a master regulator of the RAS-ERK pathway activity and cellular response, we decided to test whether methylation reactions could be considered as an effective treatment strategy in melanoma. It has been described that *MTAP* gene is frequently homozigously co-deleted along with *CDKN2A* in melanoma (Bishop et al., 2009), exerting a tumor-promoting effect including induction of invasiveness, enhanced cell proliferation and resistance to cytokines. *MTAP* loss results in higher intra and extracellular methylthioadenosine (MTA) levels (Savarese et al., 1983) that might modulate cellular responses *in vivo*. Although the loss of *MTAP* expression may play a role in the development of melanomas (Behrmann et al., 2003), the molecular link between loss of *MTAP*, enhanced MTA accumulation and induction of cellular invasion

is not known. Our results show that MTA effectively inhibits cell proliferation and viability *in vitro*. We have also found that MTA presents a potent cytostatic effect and it is capable of inhibiting tumoral growth *in vivo* in our mouse melanoma xenograft model.

As we have described before, MTA is a potent inhibitor of methyltransferases and it induces a wide variety of cellular responses: while in the nano molar (nM) range MTA has been shown to have a tumor-supporting role (Stevens et al., 2009), micro molar (μM) concentrations can interfere with cell proliferation, tumor development, invasiveness and control of apoptosis (Ansorena et al., 2002; Chen et al., 2007; Pascale et al., 2002). MTA inhibits mitogen-induced proliferative response in liver and colon cancer cells (Ansorena et al., 2002; Li et al., 2009) and has proapoptotic effects in liver cancer cells by selectively inducing Bcl-x5 (Yang et al., 2004). The aim of our research was to test the therapeutic potential of MTA in melanoma and to assess whether MTA presents proapoptotic or cytostatic properties in melanoma cancer cells. In agreement with previous results showing the inhibitory effects of MTA in proliferation and invasion, the treatment of a panel of melanoma cells with different concentrations of MTA in the μM range showed that MTA effectively inhibits cell proliferation. All melanoma cell lines were significantly more sensitive to MTA than normal MEFs or normal melanocytes. Cells harboring $BRAF^{V600E}$ mutation were more sensitive to MTA and showed a lower proliferation rate than that wild type or $NRAS^{Q61L}$ mutant cells. It is not known why BRAF mutant cells are more sensitive to MTA treatment, however it is known that the genetic signature within tumor cells can condition the drug response: we can hypothesize that

the antiproliferative MTA capabilities are enhanced in cells addicted to oncogenes with potent mitogenic effect. In addition to this, both BRAF mutant cell lines used in our experiments harbored a deletion in *CDKN2A* gene and very low expression of MTAP enzyme, which might explain the enhanced sensitivity to MTA treatment.

Previous reports have suggested that MTA might have prop-apoptotic properties in certain tumor cells (Ansorena et al., 2002) while several studies have shown that MTA inhibits proliferation in Raji and T-lymphoma cells (Nishikawa et al., 1987; Yamanaka et al., 1987) exerting a cytostatic effect. In our research we found that long-term treatment with MTA induced the dephosphorylation of the downstream mTOR target ribosomal S6 protein and correlated with a decrease in cyclin D1 levels. MTA treatment also induced a cell cycle halt in G1 phase and we were not able to observe any molecular indicator of apoptosis, suggesting that the antiproliferative capabilities exhibited by MTA correspond to a cytostatic rather that proapoptotic effect. Interestingly, the higher accumulation of cells in G1 phase corresponded to one of the BRAF mutant cell line (UACC903), which is in agreement with the proliferation results.

Interestingly, the tumor proliferation inhibitory properties of MTA were also effective *in vivo* with non-detectable side effects: MTA treatment reduced *in vivo* tumor growth by 45% where MTA treated tumors only showed a slight increase in the number of apoptotic cells compared with control tumors. However, in agreement with our *in vitro* results, we observed that tumors treated with MTA presented lower rates of proliferation according to Ki67 and cyclin D1 markers. In

addition to this, MTA treated tumors also showed low levels of VEGF expression, and a reduced number of vessels, suggesting an antiangiogenic role for MTA. Previous reports have shown that MTA is a non-toxic compound (ID50 for intramuscular injected rats: 2.9+0.4 g/kg) and is stable at physiologic pH and temperature (Simile et al., 2001), presenting MTA as a valuable agent with antitumoral capabilities.

In summary our results show the therapeutic possibilities of the natural occurring nucleoside MTA. We found that MTA inhibits melanoma cell proliferation and *in vivo* tumoral growth, supporting the antitumoral potential showed by other authors in other tumor types. We highlight the importance of MTA and present it as a therapeutic candidate for melanoma treatment.

Conclusions

1. MTA, a potent inhibitor of methyltransferase activity, increases ERK signal amplitude in response to growth factors. This effect is ligand dependent and pathway specific.

2. PRMT5, a protein arginine methyl transferase, binds and methylates RAF proteins *in vivo* and *in vitro*.

3. PRMT5 exerted methylation process modulates RAF kinase activity.

4. PRMT5 catalytic activity is dependent of growth factors.

5. Inhibition of methylation stabilizes RAF proteins and increases RAF proteins kinase activity.

6. In PC12, Inhibition of protein methylation switches the response to growth factors from proliferation to differentiation.

7. MTA inhibits melanoma cell proliferation and survival.

8. MTA inhibits tumoral growth *in vivo.*

V. SUMMARY IN SPANISH

V. RESUMEN EN CASTELLANO

I. INTRODUCCIÓN

1. Sobre el cáncer

Según la Organización Mundial de la Salud (OMS), el cáncer es la principal causa de muerte en el mundo. Se estima que en el año 2030 el número total de muertes por cáncer alcance los 11 millones anuales.

Las células en los organismos multicelulares deben organizarse y mantener un control estricto y preciso de procesos como la proliferación, la división celular o la diferenciación para mantener el equilibrio adecuado entre nacimiento y muerte celular. Cuando los mecanismos naturales de control se alteran, el equilibrio que regula la proliferación o muerte de las células desaparece y se adquiere un fenotipo tumoral. Aunque es difícil definir un célula tumoral, los tumores se pueden reconocer porque las células que los conforman presentan patrones de crecimiento anormal sin ningún sistema que las controle.

2. Señalización celular en cáncer

Se entiende por señalización celular al sistema de control que regula las actividades y acciones de las células. Las vías de señalización celular son importantes no sólo para entender los fenómenos de progresión tumoral sino cualquier proceso celular incluyendo proliferación, apoptosis, migración o angiogénesis (Helfrich et al.,

2010). Durante los procesos tumorales, estas vías de señalización se pueden encontrar hiperactivadas o silenciadas, resultando en una transmisión incorrecta de las señales celulares y, por ende, en el inicio del proceso tumoral.

2.1 Proteínas quinasas activadas por mitógenos (MAPK) y cáncer

Unas de las vías de señalización mejor estudiadas es la de las MAPKs. Todas las células eucariotas poseen MAPK que regulan la expresión génica, la división celular, controlan el metabolismo y la movilidad así como los procesos de diferenciación y apoptosis. El grupo de MAPKs más conocido corresponde a la cascada de señalización RAS-ERK. Esta vía consta de tres componentes principales, RAF, MEK y ERK que se fosforilan de manera secuencial para integrar los estímulos extracelulares en señales que llegan al núcleo e inician una respuesta celular.

ERK fue la primera MAPK caracterizada y se expresa virtualmente en todos los tejidos. La cascada de señalización de ERK comienza cuando un ligando, como un factor de crecimiento, se une a su receptor tirosina quinasa (RTK), cuya dimerización inicia la actividad quinasa de la vía y la cascada de fosforilación siguiente. Una vez activado, ERK se transloca al núcleo donde fosforila substratos específicos implicados en la regulación de varias respuestas celulares. La vía RAS-ERK tiene un papel fundamental en cáncer, ya que RAS está mutado en el 15% de los cánceres humanos (Davies et al., 2002) y el

30% de todos los cánceres presentan activaciones en ERK (Allen et al., 2003).

Por otro lado, las proteínas RAF (ARAF, BRAF, CRAF) han sido estudiadas extensamente como efectores de la señalización iniciada por RAS. La mayoría de las mutaciones conocidas en BRAF son activadoras y se caracterizan por ser capaces de estimular la señalización de ERK (Davies et al., 2002). Estas mutaciones activadoras suelen ocurrir en residuos con actividad catalítica que está altamente conservados y por tanto presentes en las otras isoformas de RAF, a pesar de lo cual no se han descrito mutaciones en CRAF o ARAF. Esto puede ser explicado en parte porque los mecanismos requeridos para la activación de ARAF o CRAF son más complejos que los de BRAF, y por tanto las mutaciones que afectan a los segmentos de activación de estas proteínas pueden no ser suficientes para inducir una conformación activa (Wellbrock et al., 2004a). CRAF por ejemplo es la isoforma de RAF mejor estudiada, pero los eventos necesarios para su activación aun no han sido comprendidos en su totalidad. Así, para que CRAF inicie su actividad quinasa se requiere un mecanismo que incluye reclutamiento a la membrana, unión a otras proteínas, homo o heterodimerización, fosforilación, etc. Teniendo lo anterior en cuenta, y dado el papel central que juega la vía RAS-ERK en la mediación y transmisión de las señales de crecimiento celular, es esencial comprender y estudiar como se modulan los diferentes componentes de esta vía para entender los mecanismos que controlan la progresión y el desarrollo del melanoma y de otros tipos de cáncer.

2.2 Proteínas arginina metil transferasas (PRMTs) en cáncer

Las vías de señalización como las MAPKs no se limitan a transmitir una señal del entorno extracelular hasta el núcleo, donde iniciará una respuesta, sino que integran y modulan esa señal para que la respuesta celular responda de manera precisa a los requerimientos de la célula. En este escenario las modificaciones post-traduccionales, como la fosforilación o la acetilación, son esenciales para el ajuste y la modulación del mecanismo de regulación de la señal celular.

A pesar de que la fosforilación es el mecanismo de modulación mejor conocido, recientemente la importancia de otras formas de modificación post-traduccional, como la metilación, está siendo ampliamente reconocida. Así, la metilación en residuos de arginina esta adquiriendo una gran relevancia como mecanismo regulatorio ya que está involucrada en procesos tales como la proliferación celular, la transcripción genética o la transducción de la señal (Bedford and Clarke, 2009).

Existen 9 proteínas, conocidas como PRMTs, capaces de metilar residuos de arginina en otras proteínas. Estas proteínas toman un grupo metilo de la molécula donadora *S*-adenosyl-L-methionine (AdoMet) y lo transfieren a los residuos de arginina de las proteínas. Como la adición de grupos metilo cambia la orientación del amino ácido y elimina potenciales puentes de hidrógeno, las modificaciones por metilación en residuos de arginina pueden modificar la capacidad de unión de las proteínas entre sí y por tanto sus funciones fisiológicas. La PRMT más conservada evolutivamente es PRMT5, que además está

involucrada en la regulación de varias vías de señalización relacionadas con la muerte celular y el proceso de transformación maligna de las células: la supresión de PRMT5 se ha relacionado con retraso en el crecimiento mientras que su sobreexpresión causa híper proliferación celular. Aunque históricamente la metilación en residuos de arginina se ha relacionado con las proteínas nucleares conocidas como histonas, recientes investigaciones demuestran que el rango de acción de las PRMTs en general y de PRMT5 en particular es mayor de lo que se pensaba. Así, se ha descrito que PRMT5 puede metilar al gen supresor de tumores p53, modificando por tanto su actividad (Chuikov et al., 2004; Huang et al., 2006; Shi et al., 2007). Además, se ha comprobado que la metilación en residuos de arginina mediada por PRMT5 es esencial en células T para el reclutamiento de factores de transcripción necesarios para la expresión génica de las citoquinas (Richard et al., 2005). A pesar del creciente número de artículos describiendo la actividad de PRMT5, la mayoría de sus funciones no son comprendidas en su totalidad: no existen modelos de ratón donde se haya anulado la expresión de PRMT5 y se desconocen las consecuencias biológicas de la sobreexpresión o reducción de los niveles de PRMT5. Por esto, es esencial determinar las funciones de PRMT5, la naturaleza de su interacción con otras proteínas y las consecuencias biológicas de la metilación en residuos de arginina.

Los estudios iniciales relacionando las PRMTs con cáncer hacían referencia a como la desregulación de la actividad de las PRMTs correlacionaba con la transformación celular. Así, se sabe los niveles de PRMT1 están más elevados en células tumorales de mama que en células normales, mientras que CARM1 se encuentra sobre expresada

en algunas formas agresivas de cáncer de pecho. Puesto que PRMT5 media en la regulación de numerosas vías de señalización celular, su relación con el cáncer es patente. Así, mientras que la supresión de PRMT5 suprime el crecimiento celular, su híper expresión está relacionada con un incremento de la proliferación. Además, PRMT5 metila al gen supresor de tumores p53 modificando su actividad. Teniendo esto en cuenta, determinar las funciones de PRMT5 y los efectos de su interacción con otras proteínas puede ser de gran interés para ampliar el conocimiento de la señalización celular en cáncer.

3. Melanoma

Dentro del conjunto de los cánceres de piel, el melanoma es la forma más letal conocida: un tumor de sólo 4 mm presenta un elevado riesgo de metástasis, y una vez diagnosticado, el melanoma metastático presenta una supervivencia media de 6-8 meses. Existen varios tipos de melanoma siendo el más común el melanoma de extensión superficial, que aparece en áreas del cuerpo con exposición intermitente al sol, como el tronco o las extremidades. A día de hoy, las únicas herramientas efectivas para luchar contra el melanoma son la detección temprana y la prevención. Además, la aparición de nuevas herramientas y los estudios genéticos están incrementando exponencialmente el conocimiento sobre el melanoma y permiten el desarrollo de nuevos fármacos y la identificación de genes cruciales para el desarrollo y evolución de la enfermedad.

3.1 MAPK en melanoma

La quinasa ERK está híper activada en el 90% de los melanomas humanos, induciendo proliferación, supervivencia, invasión y angiogénesis de las células tumorales en melanoma. En consonancia con la estrecha relación de las MAPK en los procesos tumorales, la familia de proto-oncogenes RAS (HRAS, NRAS y KRAS), se encuentra implicada con frecuencia en melanoma donde suelen aparecer sobre expresados o mutados. Así, mutaciones activadoras de NRAS están presentes en el desarrollo de nevus congénitos, melanomas primarios y metastáticos (Demunter et al., 2001), mientras que alteraciones en HRAS han sido descritas en otros tipos tumorales y presentan la habilidad de transformar melanocitos normales (Bastian et al., 2000).

Por otra parte, BRAF es un potente activador de ERK y sus mutaciones son el evento genético más relevante en melanoma. El 95% de las mutaciones en BRAF afectan a la valina en la posición 600, resultando en una activación constitutiva de la actividad quinasa de BRAF, que a su vez híper estimula a MEK. A pesar de la importancia de BRAF en el desarrollo del melanoma, la lista de oncogenes y genes supresores de tumores que contribuyen al desarrollo y mantenimiento del melanoma está lejos de ser completada. Debido a esto, estudiar los mecanismos que gobiernan la señalización de MAPK en melanoma es esencial para entender los mecanismos que regulan la progresión de esta enfermedad.

3.2 Genética del Melanoma: el locus 9p21

La región cromosómica 9p21 es un lugar de sumo interés en el estudio de todos los cánceres puesto que deleciones homocigóticas del gen *CDKN2A* se han observado en líneas celulares tumorales de muy diversos tipos (Nobori et al., 1994). Además, mutaciones en este gen suelen correlacionar con un alto riesgo de desarrollar melanoma. El gen *CDKN2A* codifica dos proteínas con actividad supresora de tumores: INK4 y ARF: la pérdida de INK4 resulta en al inactivación de la proteína del retinoblastoma (RB), alterando la progresión natural de ciclo celular (Serrano et al., 1993). Por otra parte, ARF es un potente supresor tumoral que estabiliza y potencia la actividad de p53. Por tanto, la pérdida de ARF inactiva p53 (Kamijo et al., 1998; Pomerantz et al., 1998; Zhang et al., 1998), un paso esencial en virtualmente todos los procesos tumorales.

A pesar de que numerosos estudios destacan la importancia del gen CDKN2A en la región cromosómica 9p21 para el desarrollo del melanoma, mutaciones en este gen son responsables solamente de una parte del total de melanomas. Esto sugiere que deben existir genes adicionales que estén implicados de forma más o menos relevante en el desarrollo y mantenimiento del melanoma. Uno de los genes que más relevancia está adquiriendo en el campo del melanoma es *MTAP*. *MTAP* codifica para la enzima metiltioadenosil fosforilasa, que regula los niveles intracelulares del nucleósido MTA, un potente inhibidor de la actividad metil transferasa en la célula. El locus de *MTAP* se encuentra en la región cromosómica 9p21, cerca del locus de *CDKN2A*. Se ha comprobado que *MTAP* se encuentra frecuentemente

delecionado en melanoma (Chin et al., 2006) y que la supresión conjunta de *MTAP* y *CKN2A* correlaciona con un incremento del riesgo de desarrollar melanoma (Bishop et al., 2009).

3.3 El gen MTAP

En células normales, MTAP cataliza la fosforilación y metabolización de MTA (metiltioadenosina), nucleósido que presenta unos potentes efectos inhibidores de la actividad metil transferasa. Además se sabe que MTA en concentraciones micro molares tiene efectos sobre la proliferación celular, invasividad y progresión tumoral y el control de la apoptosis (Ansorena et al., 2002; Chen et al., 2007; Pascale et al., 2002). Curiosamente, aunque estos efectos se han descrito en una gran variedad de líneas y procesos tumorales, no hay datos referentes al melanoma. Como la pérdida frecuente de la expresión de *MTAP* en melanoma se relaciona directamente con un incremento intracelular de MTA, varias preguntas sobre los efectos de la acumulación de MTA permanecen sin aclarar: (1) ¿Cuáles son las consecuencias de la pérdida de MTAP y la acumulación de MTA? (2) ¿Tiene MTA propiedades anti proliferativas en las células tumorales de melanoma? (3) ¿La acumulación de MTA puede afectar de alguna manera a las vías de señalización (como MAPKs) implicadas en el desarrollo y mantenimiento del melanoma?

II. OBJETIVOS DE LA INVESTIGACIÓN

Teniendo en cuenta los datos anteriormente expuestos, los objetivos que nos planteamos antes de iniciar nuestra investigación fueron los siguientes:

1. Investigar los efectos de MTA en células de melanoma.
2. Identificar los mecanismos moleculares relacionados con los efectos de MTA.
3. Determinar el posible potencial terapéutico de MTA como agente antitumoral en el tratamiento del melanoma.

III. RESULTADOS Y DISCUSIÓN

En muchas líneas celulares de melanoma la pérdida del locus de MTAP es una deleción frecuente que correlaciona con el aumento de los niveles intra y extra celulares de MTA. Varios estudios han demostrado que elevadas concentraciones de MTA, que es un potente inhibidor de la actividad metil transferasa, pueden interferir con proliferación celular, los mecanismos de control de la apoptosis, la invasividad y el desarrollo tumoral. A pesar de estos datos, no existe demasiada información relacionando la pérdida de MTAP y la acumulación de MTA con los posibles efectos que esto puede ocasionar en las vías de señalización que regulan la transducción de la señal en melanoma. Debido a esto, durante mi tesis hemos decidido investigar los efectos de la adición de MTA sobre la señalización celular y hemos demostrado por primera vez que la metilación en residuos de

arginina es capaz de alterar la actividad quinasa de la vía RAS-ERK en respuesta a factores de crecimiento. Hemos descubierto que PRMT5 es la metil transferasa implicada en este proceso y que la metilación controla la estabilidad de las proteínas RAF y, por ende, la actividad de las mismas. Además demostramos que la inhibición de los procesos de metilación es capaz de modular la respuesta celular inducida por los factores de crecimiento, pasando de la inducción de la proliferación a la diferenciación.

Nuestros resultados indican que la adición de MTA a células de melanoma es capaz de incrementar el nivel de fosforilación de ERK en respuesta a factores de crecimiento. Ya que factores de crecimiento como HGF son capaces de activar también la vía PI3K-AKT de forma dependiente de RAS, estudiamos el estado de fosforilación de AKT tras la adición de MTA y comprobamos que no había cambios de ningún tipo, indicando que el incremento de fosforilación en ERK inducido por MTA es exclusivo de la vía de RAS-ERK y dependiente de la activación con factores de crecimiento.

Puesto que MTA es un potente inhibidor de la reacciones de metilación, nuestro siguiente paso fue investigar qué mecanismo basado en la metilación era responsable del incremento de fosforilación observado en ERK tras la adición de MTA. Existen varios tipos de procesos de metilación que pueden modificar post-traduccionalmente las proteínas: la metilación en residuos carboxilos es una modificación necesaria para la translocación de RAS a la membrana y su posterior activación. Como nuestros resultados no mostraron alteraciones en la translocación de RAS después de la

adición de MTA, decidimos estudiar el otro gran grupo de procesos de metilación en la célula: la metilación en los grupos amino de las proteínas. Esta tipo de metilación puede tener lugar en residuos de arginina o de lisina. Puesto que la vía de RAS-ERK tiene todos sus componentes en el citoplasma descartamos la metilación en residuos de lisina porque este proceso está restringido principalmente a las histonas nucleares. Por otra parte, la metilación en residuos de arginina en un proceso eminentemente citoplasmático. Existen nueve proteínas (PRMTs) capaces de metilar en residuos de arginina. La mejor estudiada es PRMT1, que es la responsable del 85% de los procesos de metilación que tienen lugar en la célula, pero nuestros resultados indicaron que PRMT5 podía ser la enzima implicada en la modulación de la fosforilación de ERK: observamos que la presencia de PRMT5 inhibe la fosforilación de ERK en respuesta a factores de crecimiento. Además encontramos que PRMT5 se une *in vitro* a CRAF y comprobamos que la fosforilación de ERK inducida por MTA desaparecía en células donde eliminamos la expresión de CRAF, indicando que CRAF era el objetivo más probable del proceso de metilación mediado por PRMT5.

Estudios previos han mostrado que PRMT5 cataliza una metilación simétrica de residuos de arginina en zonas de las proteínas donde la secuencia es rica en motivos glicina-arginina-glicina (GRG). Esta secuencia, que indica que una proteína es potencialmente metilable, está evolutivamente conservada en todas las isoformas de RAF. Teniendo esto en cuenta, decidimos investigar el papel de esta secuencia en el proceso de metilación y diseñamos dos péptidos sintéticos que contenían respectivamente la secuencia GRG o una

versión mutada de la misma donde sustituimos la arginina central por una lisina (GKG). Realizamos ensayos de metilación *in vitro* usando PRMT5 como fuente de actividad metil transferasa y observamos que el péptido GRG se metilaba intensamente mientras que el péptido mutante GKG no presentaba signo alguno de metilación. Además, realizamos varios experimentos de metilación *in vivo* que confirmaron la identidad de este residuo de arginina, correspondiente a la posición 563 en la secuencia de CRAF y a la 671 en la de BRAF: nuestros experimentos indicaron que la forma mutante de CRAF donde la arginina 563 se sustituye por una lisina ($CRAF^{R563K}$) es incapaz de ser metilada. Más aún, la eliminación de PRMT5 mediante el uso de RNA de interferencia eliminó por completo la metilación de CRAF *in vivo*. Todos estos datos sugieren que PRMT5, en respuesta a factores de crecimiento, es capaz de metilar CRAF en la arginina 563. Además, fuimos capaces de observar que el proceso de metilación mediado por PRMT5 modifica la actividad quinasa de CRAF, inhibiéndola, lo que explica el incremento en la fosforilación de ERK cuando se suprimen los procesos de metilación al tratar con MTA o al eliminar PRMT5.

De acuerdo con la habilidad de PRMT5 de limitar el estado de fosforilación de ERK, observamos que la eliminación de PRMT5 y el uso del mutante de CRAF (R563K) que no puede ser metilado incrementaba la estabilidad de la proteína, sugiriendo que el proceso de metilación disminuía la estabilidad de RAF y lo redireccionaba al proteosoma. Para comprobar esta hipótesis realizamos una serie de experimentos para medir la vida media de CRAF antes y después de inhibir la actividad metiltransferasa: nuestros resultados indicaron que el uso de MTA o la eliminación de PRMT5 incrementaban la vida media

de CRAF en un 50% y como consecuencia la actividad quinasa de CRAF era mayor. Además observamos que tras la supresión de los procesos de metilación había un acúmulo de la población de CRAF fosforilado en la serina 621, que se ha correlacionado con la estabilidad de CRAF al evitar su degradación en el proteosoma. Todos estos datos sugieren que el proceso de metilación mediado por PRMT5 en respuesta a factores de crecimiento puede modular la intensidad de la señal (nivel de fosforilación de ERK) y la actividad quinasa de las proteínas RAF regulando la estabilidad de las mismas. Es interesante comentar que la metilación puede afectar a la forma en que las proteínas interaccionan entre sí: la adición de grupos metilos en los hidrógenos de los grupos guanidino de la arginina no modifica la carga del amino ácido pero sí puede afectar a la capacidad de la proteína de formar puentes de hidrógeno con otras proteínas. Así, podemos hipotetizar que la inhibición de la metilación correlaciona con un incremento en la formación de heterodímeros CRAF-BRAF, que poseen mayor actividad quinasa que sus correspondientes monómeros u homodímeros. En este escenario, la metilación en las proteínas RAF no sólo controlaría la estabilidad de las mismas sino que podría regular también su habilidad para formar inmuno complejos, modulando así su actividad enzimática.

Todos los procesos celulares, desde la diferenciación a la apoptosis dependen de la actuación de vías de transducción de la señal. El nivel de complejidad de estas vías, como la cascada de señalización RAS-ERK, es altamente elevado y aún hoy los eventos necesarios para la activación de estas vías no se comprenden en su totalidad. Así, la cascada RAS-ERK puede ser activada por diferentes factores de crecimiento que determinarán respuestas celulares diferentes. Además,

los componentes de la vía pueden ser alterados y/o modulados por diferentes mecanismos que afectarán a la transmisión de la señal. Estos mecanismos son modificaciones post transcripcionales como fosforilación o acetilación y, más recientemente, metilación. Teniendo esto en cuenta, tratamos de validar la hipótesis de que la metilación en CRAF es una modificación post transduccional clave en la respuesta celular iniciada por factores de crecimiento. Para comprobar esto decidimos trabajar con la línea celular tumoral PC12: en estas células los factores de crecimiento EGF y NGF son capaces de inducir la activación de ERK de modo diferencial y por tanto de iniciar respuestas celulares diferentes. Así, el tratamiento con EGF inicia una activación transitoria de ERK y las células proliferan. Por otro parte, la estimulación con NGF activa a ERK de forma sostenida y las células se diferencian. Nuestros experimentos demostraron que el tratamiento con EGF junto con el inhibidor de la metilación MTA indujo la diferenciación celular de forma completamente indistinguible de la inducida por NGF. Es decir, EGF en combinación con MTA emula por completo la respuesta iniciada por NGF. Además nuestros resultados indicaron que tanto la transfección de un mutante de PRMT5 incapaz de metilar (PRMT5ΔGAGRG) como la del mutante de CRAF ($CRAF^{R563K}$) que no puede ser metilado indujeron diferenciación celular en respuesta a EGF. En nuestro modelo con la línea celular PC12, la ausencia de PRMT5 correlaciona con un incremento en la fosforilación de ERK en respuesta a EGF, que inicia la diferenciación de las células en lugar de su proliferación. Así, hemos demostrado por primera vez como la metilación en residuos de arginina es capaz de regular la actividad quinasa de la vía RAS-ERK en respuesta a factores de crecimiento y mostramos como PRMT5 es capaza de metilar a las

proteínas RAF, modulando su actividad quinasa y limitando la amplitud de la señal en esta cascada de señalización celular.

Como nuestros resultados han mostrado que la metilación en residuos de arginina es un proceso regulador fundamental de la vía RAS-ERK, decidimos investigar a continuación si las reacciones de metilación pueden ser un objetivo terapéutico efectivo en el tratamiento contra el melanoma. A pesar de que se sabe que la pérdida de MTAP correlaciona con un aumento de las cantidades de MTA dentro de la célula, no existe información referente, en melanoma, a cómo la acumulación de MTA puede afectar al comportamiento celular y a las vías de señalización implicadas en la regulación del melanoma.

MTA es un potente inhibidor de las metil transferasas y se ha descrito que puede interferir, en células de cáncer de colon o de hígado, con la proliferación celular, la invasividad, el control de la apoptosis o el desarrollo tumoral. En nuestra investigación hemos tratado de determinar si MTA presenta propiedades pro apoptóticas o anti proliferativas. Así, de acuerdo con publicaciones previas, nuestros resultados muestran que MTA es capaz de inhibir de forma efectiva la proliferación de células de melanoma. Además observamos que las células de melanoma con mutaciones en BRAF fueron más sensibles al tratamiento con MTA que las células mutadas en RAS o no mutadas. Aunque desconocemos la causa de este comportamiento, podemos hipotetizar que las propiedades anti proliferativas del MTA se ven aumentadas en células adictas a oncogenes con actividad mitogénica, como BRAF.

A pesar de que algunas investigaciones anteriores han mostrado que el uso de MTA puede inducir apoptosis, nuestros resultados en células de melanoma indicaron que tras la adición de MTA las células se paraban en fase G1 y no fuimos capaces de identificar ninguno de los marcadores típicos de apoptosis, sugiriendo que las propiedades anti proliferativas mostradas por MTA son debidas a un efecto citostático en lugar de pro apoptótico. Además, MTA se mostró como un efectivo agente anti proliferativo en nuestro modelo de ratón *in vivo* y no apreciamos ningún efecto secundario: el tratamiento con MTA redujo el crecimiento tumoral en un 45%. En consonancia con nuestros resultados *in vitro*, los tumores tratados con MTA presentaron menores tasas de proliferación y sólo un ligero incremento en el número de células apoptóticas. Además, los tumores tratados con MTA mostraron bajos niveles de VEGF y una vascularización limitada, sugiriendo un posible papel anti angiogénico del MTA.

En resumen, nuestros resultados muestran que el uso de MTA es capaz de inhibir la proliferación celular y el crecimiento tumoral y resaltamos la importancia del MTA como un candidato terapéutico para el tratamiento del melanoma.

IV. CONCLUSIONES

1. MTA es un potente inhibidor de la actividad metil transferasa capaz de incrementar la amplitud de la señal de ERK en respuesta a factores de crecimiento.

2. PRMT5 es una proteína metil transferasa en residuos de arginina que se une y metila a las proteínas RAF.

3. El proceso de metilación mediado por PRMT5 modula la actividad quinasa de las proteínas RAF.

4. La actividad catalítica de PRMT5 es dependiente de factores de crecimiento.

5. La inhibición de la metilación estabiliza las proteínas RAF y aumenta la actividad quinasa de las mismas.

6. En el modelo de células PC12, la inhibición de la metilación es capaz de cambiar la respuesta celular, de proliferación a diferenciación, inducida por el factor de crecimiento epidérmico.

7. MTA es capaz de inhibir la proliferación celular y la supervivencia de líneas celulares de melanoma.

8. MTA inhibe el crecimiento tumoral *in vivo*.

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VII. PUBLICATIONS

1. **Protein arginine methyltransferase 5 regulates ERK1/2 signal transduction amplitude and cell fate through CRAF.**

   La proteina arginina metil transferasa 5 regula la amplitud de la señal de ERK1/2 y el destino celular a través de CRAF.

La vía de señalización celular RAS-ERK es un mecanismo esencial de la transmisión de señales moleculares que controlan la proliferación celular, diferenciación y supervivencia de las células. A pesar de que numerosos factores de crecimiento pueden activar esta vía, sus efectos sobre la amplitud y duración de la señal transmitida varían, dando como resultado respuestas celulares diferentes. Por ejemplo, en la línea celular PC12, el factor de crecimiento nervioso (NGF) inicia un incremento en la fosforilación de ERK largo y sostenido que estimula la diferenciación celular. Por otro lado, si la vía es estimulada con el factor de crecimiento epidérmico (EGF) la inducción de la fosforilación de ERK es de menor duración pero de mayor intensidad, estimulando la proliferación celular. En nuestro artículo mostramos que la metilación en residuos de arginina de ciertos componentes de la vía RAS-ERK limita la fosforilación de ERK y por tanto el tipo de señal transmitido en respuesta a factores de crecimiento. Además hemos descubierto que la restricción en la fosforilación de ERK depende de la metilación de las proteínas RAF en un proceso mediado por la proteína metiltransferasa PRMT5. Hemos comprobado que la metilación dependiente de PRMT5 de moléculas activas de las proteínas BRAF y CRAF incrementa la degradación de las mismas y por tanto reduce su actividad catalítica. La inhibición de PRMT5 o la expresión de mutantes de RAF que no pueden ser metilados no sólo aumenta la amplitud y la

duración de la fosforilación de ERK en respuesta a factores de crecimiento, sino que es capaz de cambiar el comportamiento de las células PC12 en respuesta a EGF, pasando de proliferación a diferenciación. En resumen, en este artículo presentamos un nuevo mecanismo de regulación dentro de la vía RAS-ERK mediante el cual la actividad kinasa de la vía puede ser regulada modulando así la respuesta celular iniciada por un factor de crecimiento.

CELL BIOLOGY

Protein Arginine Methyltransferase 5 Regulates ERK1/2 Signal Transduction Amplitude and Cell Fate Through CRAF

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The RAS to extracellular signal–regulated kinase (ERK) signal transduction cascade is crucial to cell proliferation, differentiation, and survival. Although numerous growth factors activate the RAS-ERK pathway, they can have different effects on the amplitude and duration of the ERK signal and, therefore, on the biological consequences. For instance, nerve growth factor, which elicits a larger and more sustained increase in ERK phosphorylation in PC12 cells than does epidermal growth factor (EGF), stimulates PC12 cell differentiation, whereas EGF stimulates PC12 cell proliferation. Here, we show that protein arginine methylation limits the ERK1/2 signal elicited by particular growth factors in different cell types from various species. We found that this restriction in ERK1/2 phosphorylation depended on methylation of RAF proteins by protein arginine methyltransferase 5 (PRMT5). PRMT5-dependent methylation enhanced the degradation of activated CRAF and BRAF, thereby reducing their catalytic activity. Inhibition of PRMT5 activity or expression of RAF mutants that could not be methylated not only affected the amplitude and duration of ERK phosphorylation in response to growth factors but also redirected the response of PC12 cells to EGF from proliferation to differentiation. This additional level of regulation within the RAS pathway may lead to the identification of new targets for therapeutic intervention.

INTRODUCTION

A major challenge in cell signaling is to understand how different external cues and cell membrane receptors give rise to unique biological responses despite their promiscuous activation of shared pathways. For instance, although various growth factors initiate signaling through the same pathways (*1*), the biological consequences of the activation of a particular signaling pathway by different growth factors may differ. Many growth factors activate receptor tyrosine kinases (RTKs) to signal through the RAS (*2*) to RAF to mitogen-activated protein kinase (MAPK) signaling pathway. The extracellular signal–regulated protein kinase 1 and 2 (ERK1/2), MAPKs activated by phosphorylation and inactivated by dephosphorylation, play a prominent role in this pathway by phosphorylating transcription factors, cytoskeletal proteins, and enzymes (including other protein kinases) (*3*). Three different quantitative measures can be used to assess kinase signaling: signal amplitude (the peak response to a stimulus), duration (is the response transient or sustained?), and integral strength (integrated concentration of an active molecule, derived from the other two measures) (*4*, *5*). From an oversimplified perspective, phosphorylation and dephosphorylation determine whether kinases are active or inactive; however, their subcellular distribution and, presumably, posttranslational modifications other than phosphorylation (*6*, *7*) will influence the final biological outcomes. Signaling through the RAS-ERK1/2 pathway can be modulated at various levels; however, the activation of specific RAF isoforms, their homo- or heterodimerization with other isoforms, and their degradation are particularly relevant not only to the activation of ERK1/2 but also to determining the amplitude, duration, and integral strength of ERK1/2 phosphorylation (*4*, *5*, *8*–*10*).

Protein arginine methylation is increasingly being recognized for its role in regulating signal transduction, RNA processing, transcriptional activation, and DNA repair (*11*–*13*). The existence of a wide range of arginine-methylated substrates suggests that this eukaryotic modification may play a role as complex as that of phosphorylation and raises the possibility that these two regulatory mechanisms are somehow coordinated. Among the nine protein arginine (R) methyltransferases (PRMTs) in humans with a demonstrated physiological enzymatic activity (PRMT1 to 9) (*11*), PRMT5 was the first determined to catalyze the formation of symmetric dimethylarginines (sDMAs) on a Gly-Arg-Gly (GRG) motif (*14*). PRMT5 has been implicated in transcriptional regulation through histone methylation (*15*, *16*) and methylation of the RNA polymerase II CTD phosphatase (FCP1) (*17*). It has also been implicated in promoting spliceosome assembly (*18*) and appears to be an HSP90 (heat shock protein 90 kD) client (*19*). Given these roles, it is unexpected that most PRMT5 is in the cytoplasm and not in the nucleus (*20*). However, PRMT5 was initially identified as a Janus kinase binding protein 1 (JBP1) (*21*), and it has also been found to interact with the death receptor for TRAIL (tumor necrosis factor–related apoptosis-inducing ligand) (*22*). Furthermore, PRMT5 is a component of the branch of the RAS signaling cascade implicated in regulating morphology in *Schizosaccharomyces pombe* and it positively modulates Shk1

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[Ste20/p21-activated kinase (PAK) homolog] function (*23*), suggesting that PRMT5 may have unappreciated cytoplasmic functions.

Although the molecular machinery by which various growth factors control signal transduction has been extensively studied (*1*), the mechanism regulating signal amplitude in response to a given stimulus is largely unknown. Here, we show that arginine methylation of RAF proteins limits the ERK1/2 phosphorylation elicited by stimulation with certain growth factors and identify PRMT5 as the protein methyltransferase responsible for fine-tuning growth factor signals. PRMT5 forms a complex with RAF proteins and methylates them, decreasing their kinase activity and stability, thereby diminishing the amplitude of the ERK1/2 signal. Finally, we show that inhibiting methylation can alter growth factor–dependent biological responses, switching the response of PC12 cells to EGF from proliferation to differentiation by increasing the signal amplitude and prolonging its duration.

RESULTS

5′-Methylthioadenosine increases ERK1/2 signal amplitude in response to hepatocyte growth factor

We observed that, in mouse melanoma cells, the methylation inhibitor 5′-methylthioadenosine (MTA) increased the degree of ERK1/2 phosphorylation in response to hepatocyte growth factor (HGF) treatment (Fig. 1A). Signaling through the HGF RTK c-Met activates both the RAS-ERK1/2 and the phosphatidylinositol 3-kinase (PI3K)–AKT pathways (*24*); however, MTA did not appear to affect AKT phosphorylation (Fig. 1, A and B). We found that MTA specifically enhanced the amplitude of HGF-mediated ERK1/2 phosphorylation in various mouse and human cell lines (see below) and rat primary hepatocytes (fig. S1). MTA's enhancement of ERK1/2 phosphorylation was observed 2 min after HGF stimulation (fig. S2A) and was still apparent 30 min after HGF stimulation (Fig. 1B). However, ERK1/2 phosphorylation began to decline 10 min after HGF treatment with or without MTA, indicating that phosphatase activity was unaltered (Fig. 1B). Indeed, activity of the nuclear phosphatase MKP1/2, which controls the amount of nuclear p-ERK1/2, increased with MTA treatment (fig. S2B). Consistent with this, we also observed that the translocation of phosphorylated ERK1/2 (p-ERK1/2) to the nucleus was intact in MTA-pretreated cells (Fig. 1, C and D).

Together, these data demonstrate that the methylation inhibitor MTA increases ERK1/2 signal amplitude in response to HGF. Furthermore, this seems to involve an increase in kinase activity upstream of ERK1/2, rather than a downstream decrease in phosphatase activity.

MTA-dependent augmentation of ERK1/2 phosphorylation is ligand-dependent and mediated by methylation inhibition

Next, we determined whether MTA enhanced the degree of ERK1/2 phosphorylation in response to other stimuli that activate the ERK1/2 signaling pathway. We found that MTA enhanced ERK1/2 phosphorylation mediated not only by treatment with HGF but also by exposure to the phorbol ester tetradecanoylphorbol-13-acetate (TPA), which activates the pathway in an RTK-independent manner, or epidermal growth factor (EGF) (showing limited amounts of p-ERK1/2 in response to EGF because of the low abundance of EGF receptors in the 37-31E melanoma cell line) (Fig. 1E). However, the ability of MTA to enhance ERK1/2 phosphorylation in response to either acidic or basic fibroblast growth factor (FGF1 and FGF2, respectively) depended on cell line (Fig. 1E and fig. S3A) and did not occur with melanocyte-stimulating hormone (MSH-α), which did not stimulate ERK1/2 phosphorylation (Fig. 1E). Similar results were obtained in human embryonic kidney (HEK) 293 cells and rat neuroblastoma (PC12) cells (fig. S3A).

This modulatory effect of MTA was also observed with MEK1/2 (mitogen-activated or extracellular signal–regulated protein kinase kinase 1 and 2), the kinases immediately upstream of ERK1/2 in the MAPK pathway (figs. S2A and S3D). Moreover, the increase in ERK1/2 phosphorylation by MTA upon growth factor stimulation was blocked by inhibition of MEK1/2 activity (Fig. 1F), suggesting that it did not involve crosstalk pathways that targeted ERK1/2 directly.

Next, we examined the effects of the protein methyltransferase inhibitors 3-deaza-adenosine (Deaza) and cycloleucine (c-leucine) (*25*) to confirm that the increase in signal amplitude in response to MTA indeed depended on inhibition of methylation (Fig. 1, F and G, and fig. S3, B to D). As expected, we did not observe any alteration in the translocation of p-ERK1/2 to the nucleus in either Deaza or c-leucine (fig. S3D). Together, these data indicate that protein methylation modulates signaling through the RAS pathway in response to specific growth factors upstream of MEK1/2.

Protein arginine methyltransferase PRMT5 limits ERK1/2 signal amplitude

Our data suggested that the machinery responsible for this modulation of RAS pathway signaling was cytoplasmic. Whereas lysine methylation is involved in a wide range of nuclear processes (*26*–*28*), protein arginine methylation has been implicated in several cytoplasmic responses (*13*). Among the members of the PRMT family found in humans, only PRMT1, 3, 5, and 8 are found in the cytoplasm (*29*). We used a combination of genetic and functional analyses to eliminate *Prmt1*, *Prmt3*, and *Prmt8* as possible candidates (fig. S4). Depletion of *Prmt5* with small interfering RNA (siRNA) reproduced the MTA-dependent increase in growth factor–dependent ERK1/2 phosphorylation in both tumor and normal cells, and reduced or eliminated the effect of MTA, suggesting that MTA targeted PRMT5 (Fig. 2A). Moreover, overexpression of a catalytically inactive *PRMT5* mutant (PRMT5ΔGAGRG) increased the degree of ERK1/2 phosphorylation in response to EGF (Fig. 2B).

Because PRMT5's ability to affect ERK1/2 phosphorylation depended on the specific ligand used to activate MAPK signaling, we examined whether PRMT5 activity was itself regulated by growth factors. We found that PRMT5 activity was regulated by growth factors, increasing with EGF, HGF, FGF1, and FGF2 treatment and slightly decreasing with nerve growth factor (NGF) (Fig. 2C). Together, these data indicate that PRMT5 modulates ERK1/2 signal amplitude in response to specific growth factors.

PRMT5 colocalizes with RAF proteins and modulates their catalytic activity

Our observations suggested that the putative PRMT5 target was within the RAS pathway and upstream of MEK1/2. MTA did not affect phosphorylation of the c-Met receptor (indicative of its activation) upon exposure to HGF (Fig. 3A), nor did it affect the amount of active RAS-GTP (guanosine triphosphate) observed after HGF treatment (Fig. 3B). We also observed that MTA modulates the ERK1/2 signal amplitude in $NRAS^{Q61L}$ mutant cells (which signal mainly through CRAF) (*30*), whereas this effect was not observed in $BRAF^{V600E}$ mutant cells, which signal independently of RAS (*30*) (fig. S5), supporting the RAS-dependent regulation of PRMT5. Moreover, HGF-dependent ERK1/2 phosphorylation was limited in cells in which *CRaf* was knocked down (fourfold less amount of p-ERK1/2), and the effect of MTA was nearly abolished (Fig. 3C), suggesting that CRAF participated in the methylation-mediated regulation of ERK1/2 activation. RAF proteins and PRMT5 are HSP90 clients (*19*, *31*), indicating that they may be part of the same protein complexes. We found that PRMT5 was constitutively

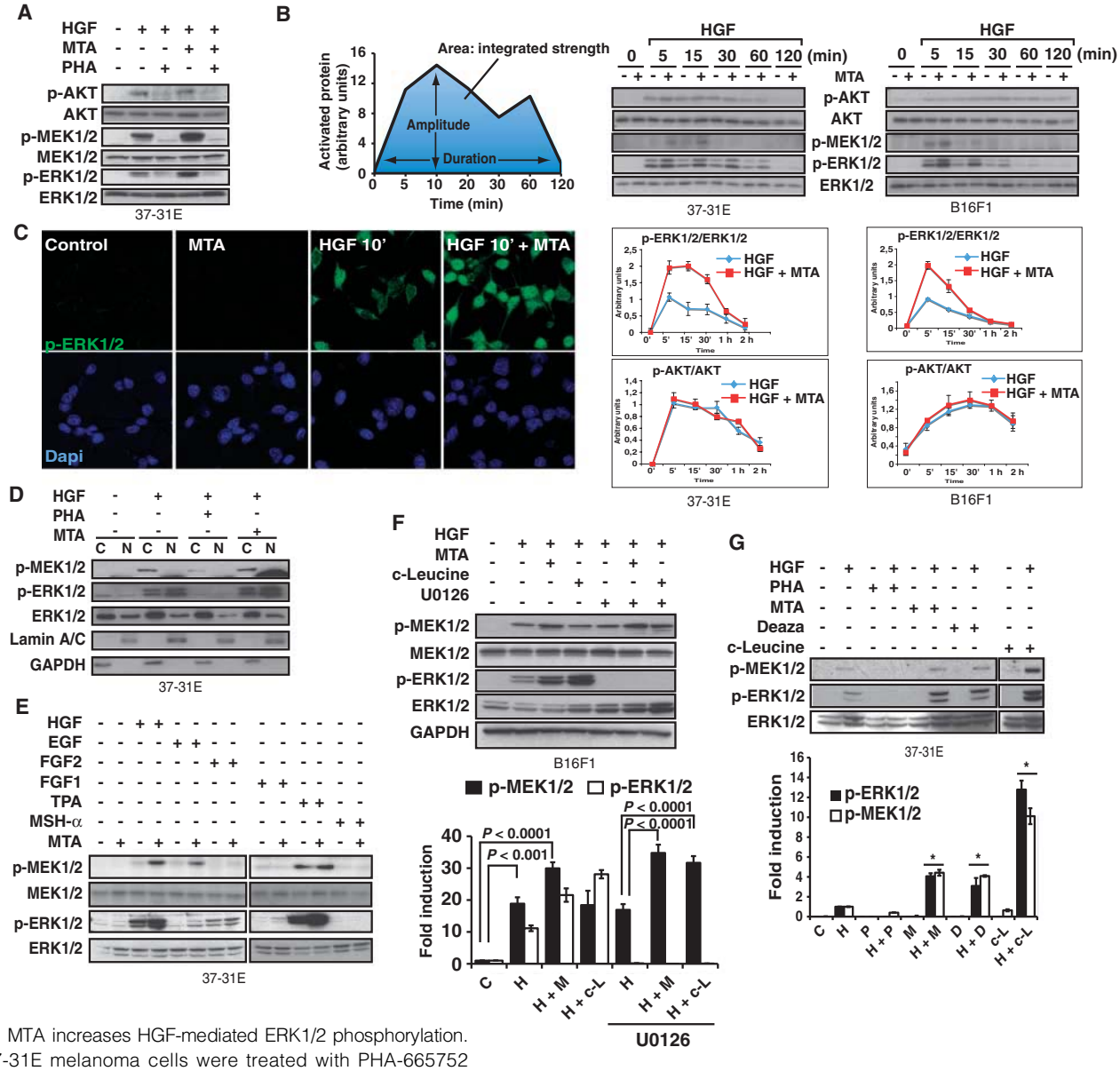

Fig. 1. MTA increases HGF-mediated ERK1/2 phosphorylation. (A) 37-31E melanoma cells were treated with PHA-665752 (PHA), MTA, or both under conditions of serum starvation, and then, after stimulation with HGF, total protein extracts were immunoblotted for p-ERK/2, ERK1/2, p-MEK1/2, MEK1/2, p-AKT, and total AKT. (B) MTA increases the HGF-dependent p-ERK1/2 signal amplitude. 37-31E and B16F1 melanoma cells were treated with MTA as in (A) and exposed to HGF for the indicated times. Graphs depict a schematic representation of the quantitative measures governing signal transduction and the quantification of the ERK1/2 and AKT activation profiles in response to HGF or HGF plus MTA from three independent experiments. (C) Immunofluorescence micrograph of p-ERK1/2 in 37-31E cells treated with HGF or MTA as indicated. (D) 37-31E cells were treated as in (A). Western blots show the cytoplasmic and nuclear distribution and phosphorylation of MEK1/2 and ERK1/2. Lamin A/C and GAPDH were used as markers of the fractions' purity. (E) MTA-mediated effect on ERK1/2 and MEK1/2 phosphorylation is growth factor–specific. 37-31E cells were treated as in (A) with the indicated growth factors for 10 min. Total ERK1/2 is shown as a loading control. One representative experiment of three is shown. (F) B16F1 cells were serum-starved and treated for 14 hours with or without MTA or c-leucine and then treated for 5 min with HGF. U0126 was applied where indicated. p-MEK1/2, MEK1/2, p-ERK1/2, ERK1/2, and GAPDH were assessed by Western blot (n = 3 experiments). (G) Methylation inhibitors increase ERK1/2 signal amplitude in response to HGF. 37-31E melanoma cells were starved and treated for 14 hours with PHA, MTA, Deaza, or c-leucine. Then, cells were exposed to HGF for 10 min. p-MEK1/2, p-ERK1/2, and total ERK1/2 are shown in one representative experiment of three. Graph shows the quantification of p-MEK1/2 and p-ERK1/2. *P < 0.05 versus HGF-treated samples; n = 3 different experiments.

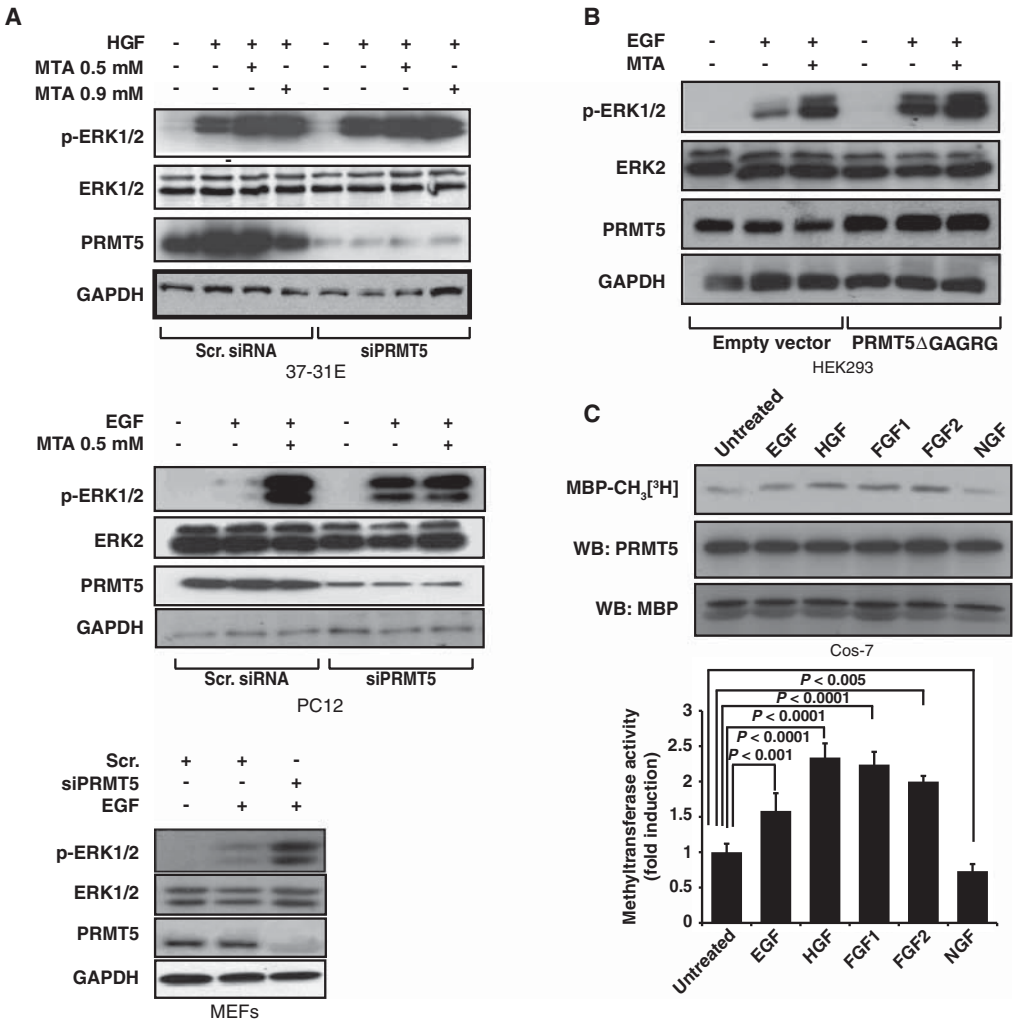

Fig. 2. *PRMT5* depletion increases ERK1/2 phosphorylation in response to growth factors. (**A**) Knockdown of *Prmt5* in 37-31E, PC12, or MEFs enhances the increase in ERK1/2 phosphorylation produced by growth factor treatment. Cells transfected with either scrambled siRNA (Scr.) or *Prmt5* siRNA were serum-starved and exposed to MTA for 3 hours and then treated with HGF for 10 min. p-ERK1/2, ERK1/2, and PRMT5 abundance was assessed by Western blot. (**B**) Overexpression of the catalytically inactive *PRMT5* mutant (PRMT5ΔGAGRG) in HEK293 cells reproduces the effects of MTA on ERK1/2 phosphorylation. Forty-eight hours after transfection with either empty vector or pEF_2-*PRMT5ΔGAGRG* expression vector, cells were serum-starved and pretreated with MTA for 2 hours and then stimulated with HGF for 10 min. Cell lysates were assessed for p-ERK1/2, ERK1/2, PRMT5, and GAPDH by Western blotting. (**C**) Growth factors regulate PRMT5 methyltransferase activity. Cos-7 cells stably transfected with Flag-*PRMT5* were serum-starved for 3 hours and then treated with the indicated growth factors for 10 min. After PRMT5 immunoprecipitation, its methyltransferase activity was measured in vitro. The ^3H radioactive signal incorporated in MBP was assessed autoradiographically after SDS-PAGE. Immunoprecipitated PRMT5 and MBP are shown as loading controls. Graph shows quantification of methyltransferase activity normalized by the amount of PRMT5 immunoprecipitated. *P* value was calculated with Student's *t* test ($n = 3$ independent replicates).

bound to CRAF and BRAF immunocomplexes (Fig. 3D and fig. S6A). The association of PRMT5 with CRAF was confirmed by immunolocalization experiments (fig. S6B). However, in vitro binding experiments using recombinant PRMT5 and recombinant full-length inactive glutathione *S*-transferase (GST)–CRAF or active GST-CRAF (N-terminal GST-tagged, residues 306 to end) showed that PRMT5 binds only to active CRAF (Fig. 3E).

To determine whether the catalytic activity of RAF family proteins was modulated by posttranslational methylation, we measured their in vitro kinase activity after immunoprecipitation from cells treated with growth factors, MTA, or both. The in vitro kinase activity of CRAF immunoprecipitated from HeLa cells transfected with Flag-$CRAF^{WT}$ and treated with HGF or EGF was significantly increased by MTA (120%; $P < 0.0001$), whereas MTA had no effect on CRAF activity in cells that had not been treated with growth factor (Fig. 3F). MTA had the same effect on endogenous CRAF immunoprecipitated from mouse embryo fibroblasts (MEFs) treated with EGF (Fig. 3G; or HeLa and HEK293 cells treated with EGF or HGF, fig. S8C). The catalytic activity of CRAF immunoprecipitated from cells treated with EGF was also increased significantly by *Prmt5* knockdown with siRNA ($P < 0.001$) (Fig. 3H). Posttranslational methylation also enhanced the in vitro catalytic activity of BRAF (figs. S7 and S8B) and ARAF (fig. S8A) immunoprecipitated from HeLa and HEK293 cells treated with growth factors. Thus, these data indicate that MTA and PRMT5 modulate the kinase activity of RAF proteins induced by growth factors.

$CRAF^{R563K}$ and $BRAF^{R671K}$ mutants are more stable than the corresponding wild-type proteins and show increased kinase activity in response to growth factors

In vivo metabolic labeling of cells showed that growth factor treatment stimulated the methylation of CRAF and BRAF and that this was prevented by MTA treatment or *Prmt5* knockdown (Fig. 4A and fig. S9A). PRMT5 methylates arginine residues within GRG motifs (*11*), and, of all components of the RAS pathway, only the RAF proteins contained a GRG motif. The GRG motif was present in all RAF family members analyzed and was conserved from *Caenorhabditis elegans* to humans (Fig. 4B). Moreover, according to the BRAF three-dimensional (3D) structure, $BRAF^{R671}$ should be available for modification by methylation (Fig. 4B).

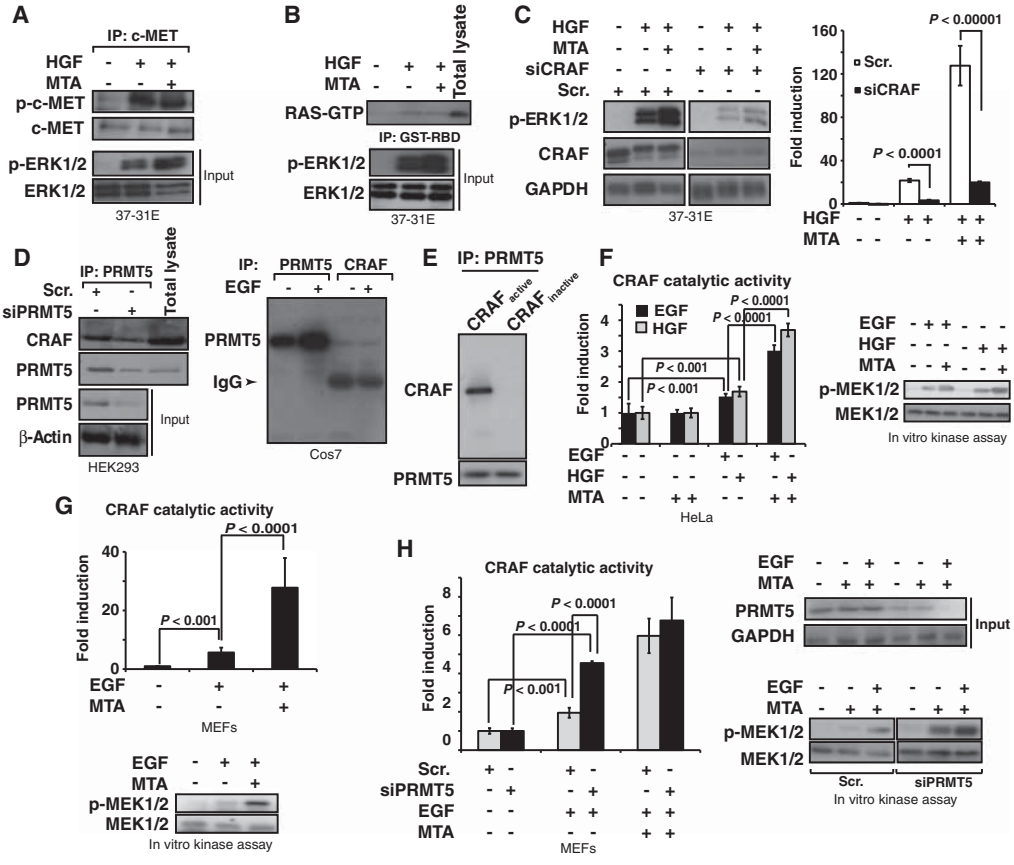

Fig. 3. MTA and PRMT5 depletion increases CRAF's catalytic activity. (**A**) 37-31E cells were serum-starved, pretreated with MTA for 3 hours, and then treated with HGF for 5 min. c-Met was immunoprecipitated from total lysates. Western blot shows the amount of p-c-Met in the immunocomplexes. p-ERK1/2 and ERK1/2 levels in the samples used in the immunoprecipitation are shown (Input). (**B**) RAS activation is not affected by MTA. 37-31E cells were treated as in (A). RAS-GTP was isolated by affinity chromatography with GST-RBD (GST–*RAS* binding domain of CRAF). Complexes were separated by SDS-PAGE. Western blot shows the amount of RAS-GTP. p-ERK1/2 and ERK1/2 demonstrate the activation of the pathway in the samples used. (**C**) 37-31E cells transfected with *CRaf* siRNA were serum-starved and pretreated with MTA for 2 hours and then triggered with HGF for 10 min. Western blot shows CRAF and p-ERK1/2. *P* value was calculated with Student's *t* test (n = 3 different experiments). (**D**) PRMT5 was immunoprecipitated from total lysates of HEK293 cells transfected with either scrambled (Scr.) or *PRMT5* siRNA. Western blots show PRMT5 and CRAF in the immunocomplexes and PRMT5 in the initial total lysates (left panel). Endogenous PRMT5 and CRAF were immunoprecipitated from lysates of Cos-7 cells untreated or treated with EGF. Western blots assess the presence of PRMT5 in the immunocomplexes. (**E**) PRMT5 binds to active CRAF. Recombinant PRMT5 was incubated with either full-length inactive GST-CRAF or active GST-RAF (N-terminal GST-tagged, residues 306 to end). Western blot shows CRAF after PRMT5 immunoprecipitation. Immunoprecipitated PRMT5 is shown as loading control. (**F**) MTA increases growth factor–induced CRAF catalytic activity. HeLa cells transiently transfected with Flag-*CRAF*^{WT} were serum-starved, treated with MTA for 3 hours, and then treated with either EGF or HGF for 10 min. Immunoprecipitated Flag-CRAF was used to perform an in vitro kinase assay. Graph shows CRAF catalytic activity (fold induction over untreated cells) in a coupled RAF-MEK-ERK-MBP radioactive assay under the different conditions (left). Western blots were performed against p-MEK under the same conditions (right). *P* value was calculated with Student's *t* test (n = 3 replicates). (**G**) MTA increases the endogenous CRAF catalytic activity in response to growth factors. MEFs were starved and treated with MTA for 3 hours, and cells were exposed to EGF for 10 min. Immunoprecipitated endogenous CRAF was used to perform an in vitro kinase assay as in (F). Graphs show the CRAF catalytic activity fold induction over untreated cells. A radioactive assay and Western blots show the p-MEK levels induced by immunoprecipitated CRAF. Bars indicate the SD. *P* value was calculated with Student's *t* test (n = 3 replicates). (**H**) 37-31E cells transfected with either scrambled (Scr.) or *Prmt5* siRNA were treated as in (F). Graph shows the kinase activity of endogenous CRAF. Western blots show the p-MEK levels induced by immunoprecipitated CRAF. PRMT5 protein levels in total lysates used in the immunoprecipitation are shown. Bars indicate the SD. *P* value was calculated with Student's *t* test (n = 3 replicates).

To investigate the role of this residue in the activation of ERK1/2 upon growth factor triggering, we generated the equivalent CRAF^{R563K} mutant. Transient transfection of the same DNA quantity of Flag-*CRAF*^{R563K} or Flag-*CRAF*^{wt} in HEK293 cells resulted in higher amounts of Flag-*CRAF*^{R563K} protein, indicating that the CRAF^{R563K} mutant was more stable than its wild-type counterpart (Fig. 4C). Cells transfected with one-sixth of the amount of Flag-*CRAF*^{R563K} compared with Flag-*CRAF*^{wt} to compensate for protein expression abundance showed more p-ERK1/2 than did Flag-*CRAF*^{wt}–transfected cells in response to EGF (14.3-fold versus 4-fold compared to Flag-*CRAF*^{wt}–transfected and untreated cells) (Fig. 4C). In agreement with this, the CRAF^{R563K} mutant showed significantly increased catalytic activity in response to the growth factor compared with that of the wild-type variant (62.9 ± 1.2% more activity; $P < 0.01$) (Fig. 4D). These results suggest that methylation of CRAF^{R563} could decrease the stability of active CRAF molecules and consequently decrease their signal output. To confirm that methylation reactions contributed to CRAF degradation upon RAS pathway activation, we measured CRAF stability in NRAS^{Q61L} mutant SKMel147 melanoma cells with or without *PRMT5* depletion or in the presence or absence of MTA (Fig. 4E and fig. S9B). Inhibition of protein methylation or *PRMT5* knockdown stabilized CRAF [~50% more stable with a half-life ($t_{1/2}$) of ~12 to 15 hours in *PRMT5* siRNA–transfected cells than in cells transfected with scrambled siRNA] (Fig. 4E). These data

correlated with the phosphorylation state of CRAF Ser^{621}, a residue whose phosphorylation prevents proteasomal degradation of CRAF (*32*) (Fig. 4E and fig. S9B). The notion that a subset of RAF molecules from the small fraction of the total pool mobilized to signal are subjected to this regulation was indirectly supported by the phosphorylation code of RAF proteins 10 min after growth factor triggering. MTA-pretreated cells compared

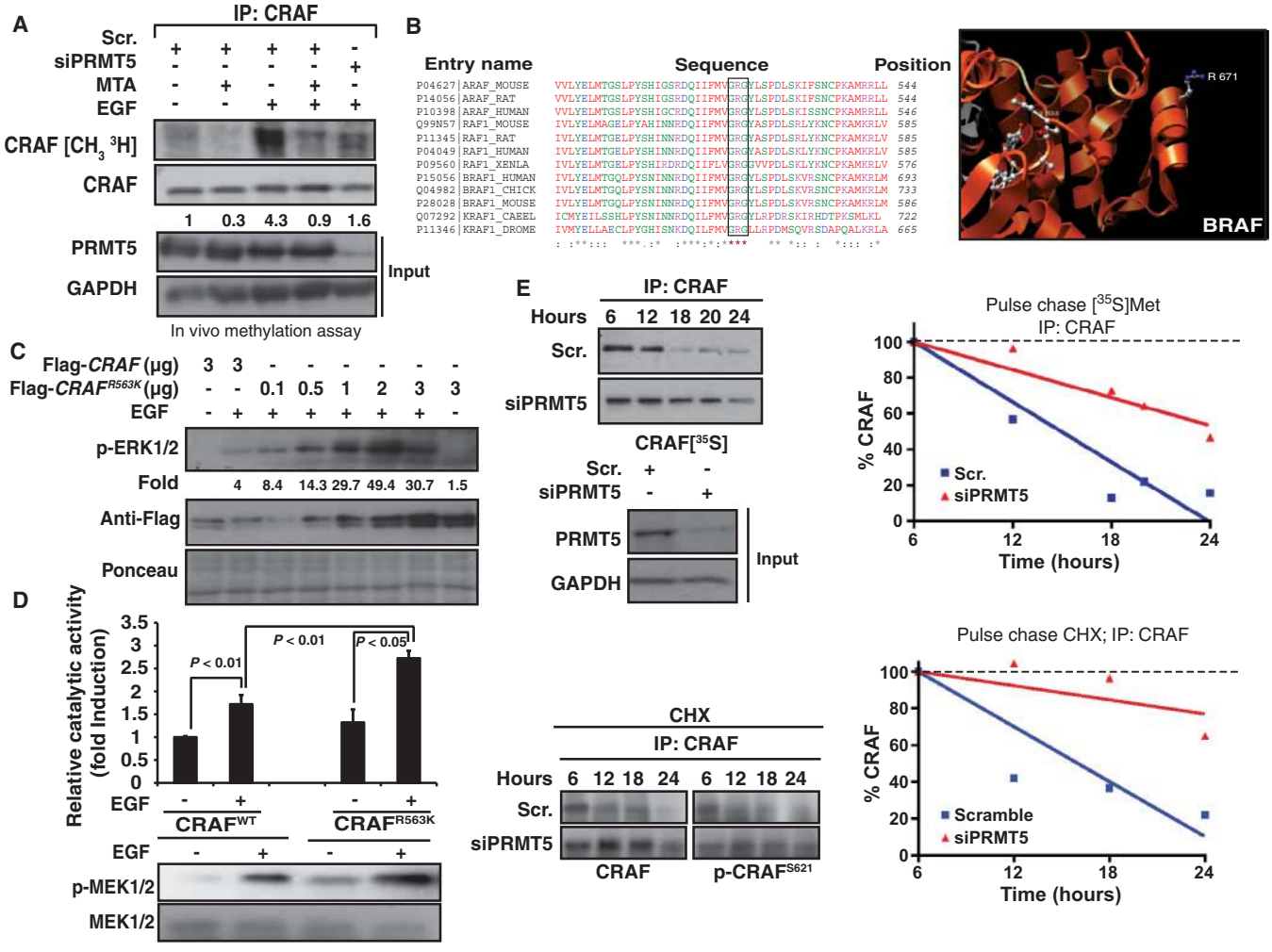

Fig. 4. Arginine-to-lysine mutation in the GRG motif of RAF proteins promotes their stability and amplifies their increase in kinase activity in response to growth factors. (**A**) In vivo methylation assay in PC12 cells. Cells transiently transfected with either scrambled or *Prmt5* siRNA and metabolically labeled ([^{3}H]methionine) were serum-starved and pretreated with MTA, and then cells were exposed to EGF for 10 min. After immunoprecipitation of endogenous CRAF, the ^{3}H radioactive signal incorporated in CRAF was assessed autoradiographically after SDS-PAGE. The same membranes were assessed for CRAF (loading control). PRMT5 in total lysates is shown in the lower panel. (**B**) Sequence alignment of RAF isoforms from different species showing the conserved GRG motif (left). A 3D image made with UCSF Chimera (http://www.cgl.ucsf.edu/chimera) and the 1UWH file from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.pdb.org) show the location of the Arg^{671} residue in the BRAF 3D structure (right). (**C**) HEK293 cells were transiently transfected with Flag-*CRAF*^{WT} or Flag-*CRAF*^{R563K} as indicated. Western blot shows p-ERK1/2 and abundance of transfected proteins. Quantification of p-ERK1/2 normalized by Ponceau S staining is shown (fold induction with respect to Flag-*CRAF*^{WT}–transfected untreated cells). (**D**) Catalytic activity of CRAF^{R563K} mutant compared to that of the wild-type isoform. HeLa cells were transfected with Flag-*CRAF*^{WT} or one-sixth of the amount of the Flag-*CRAF*^{R563K} mutant. CRAF catalytic activity was assessed in immunoprecipitates. Graph shows the CRAF catalytic activity (fold induction over untreated cells) in a coupled RAF-MEK-ERK-MBP radioactive assay under the different conditions (top). *P* value was calculated with Student's *t* test (*n* = 3 replicates). Western blots show p-MEK under the same conditions (lower panel). (**E**) SKMel147 cells were transiently transfected with scrambled siRNA (Scr.) or *PRMT5* siRNA (siPRMT5) for 24 hours. Cells were pulse-labeled with [^{35}S]Met and re-collected over a 24-hour time course (upper left panels). Cell lysates were harvested and immunoprecipitated with antibody directed against CRAF; after SDS-PAGE separation and capture of the ^{35}S signal, CRAF was quantitated by autoradiography. Left, data presented as autoradiogram; right, data presented graphically. Cells were treated with CHX (5 μg/ml) and collected at the indicated time points (lower panel). Immunoprecipitation of CRAF was performed from 500 μg of total protein. Western blots show CRAF and p-CRAF^{S621}. Graph shows CRAF quantifications. Dashed line represents 100%. Amounts of PRMT5 are shown in the input samples.

with cells treated only with growth factor showed increased amounts of p-CRAF^{S338}, a residue related to its active conformation; decreased amounts of p-CRAF^{S289,296,301}, residues related to the inactivation of CRAF; and increased amounts of p-CRAF^{S621} (fig. S9C). Similarly, the BRAF^{R671K} mutant had increased kinase activity and stability compared to wild-type BRAF, and depletion of *PRMT5* increased the stability of the wild-type protein (fig. S10, A to C). *PRMT5* knockdown did not affect ARAF stability in SKMel147 cells (fig. S10D), although ARAF's catalytic activity in response to EGF and HGF was increased by MTA in HEK293 and HeLa cells (fig. S8A).

Together, these results indicate that the arginine residue within the GRG motif regulates the kinase activity and stability of activated BRAF and CRAF and consequently modulates ERK1/2 signal amplitude.

PRMT5 methylates CRAF^{R563} after growth factor treatment

Our data suggested that CRAF was methylated on the arginine within the GRG motif. Only a small fraction of the total RAF proteins in a cell participates in signaling response to a given stimulus (fig. S11A) (*33*). To identify methylated molecules and determine whether CRAF was methylated on CRAF^{R563K}, we enriched the population of activated CRAF proteins by performing two consecutive immunoprecipitations. We transfected HeLa cells with either Flag-*CRAF^{WT}* or Flag-*CRAF^{R563K}* and, after treating them with EGF in the presence or absence of MTA, immunoprecipitated CRAF with an anti-Flag antibody. The resulting immunocomplexes were eluted with a Flag peptide, and a second immunoprecipitation was performed with an antibody directed against anti–symmetric methylarginines (SYM10). Then, Western blot and mass spectrometry analyses were used to detect CRAF in the anti-methylarginine immunocomplexes.

EGF treatment increased the amount of CRAF detected by Western blot analysis in the anti-methylarginine immunocomplexes, whereas treatment with the combination of EGF and MTA decreased it (Fig. 5A). We could not detect CRAF in anti-methylarginine immunocomplexes from cells transfected with Flag-*CRAF^{R563K}*. We scaled up the experiment to identify CRAF by mass spectrometry. Analysis of the immunoprecipitated proteins within the band showing an appropriate molecular size confirmed the presence of CRAF and also identified PRMT5 (~70 kD) (Fig. 5A). *PRMT5* knockdown abolished the EGF-mediated increase in CRAF abundance in the immunocomplexes (Fig. 5B). The identity of the methylated residue was confirmed by in vivo methylation experiments performed on HeLa and HEK293 cells and showed that the Flag-*CRAF^{R563K}* mutant was not methylated in response to growth factor treatment (Fig. 5C). Furthermore, in vitro methylation assays showed that PRMT5 methylated Arg^{563} of a CRAF-derived peptide containing the GRG (Fig. 5D), which differs from the comparable regions of ARAF and BRAF by a single amino acid (fig. S11B). Together, these data indicate that growth factors stimulate methylation of CRAF^{R563} by PRMT5.

Inhibition of methylation switches the response to growth factors from proliferation to differentiation

Both NGF and EGF stimulate the RAS-MAPK pathway in PC12 cells, but they have different effects on ERK1/2 activation: EGF elicits a transient phosphorylation of ERK1/2, whereas NGF elicits a sustained activation of ERK1/2 (*34*). This differential activation of ERK1/2 by NGF or EGF translates into distinct biological responses: NGF promotes PC12 cell differentiation, whereas EGF promotes their proliferation (*35*). We wondered whether the restriction in ERK1/2 signal amplitude and integral strength after PRMT-mediated methylation could affect cellular behavior in response to a particular ligand. To test this, we attempted to mimic the differentiating effect of NGF on PC12 cells with EGF in combination with a methyltransferase inhibitor. As previously noted, both MTA and *Prmt5* deletion enhanced EGF-mediated ERK1/2 phosphorylation in PC12 cells (Fig. 2A and fig. S3A). When we compared the effects of EGF, NGF, and EGF plus MTA on ERK1/2 phosphorylation in PC12 cells over a 3-hour time course, we observed that EGF induced a transient phosphorylation of ERK1/2, whereas NGF elicited a sustained signal with a total signal strength 3.2 ± 0.1–fold higher than that induced by EGF ($P < 0.0001$). MTA pretreatment of EGF-treated cells induced an increase in ERK1/2 signal amplitude (2.1 ± 0.9–fold versus EGF) with an integrated signal strength 2.21 ± 0.95–fold higher than that induced by EGF alone ($P < 0.0001$) (Fig. 6A). Furthermore, PC12 cells treated with EGF plus MTA differentiated in a manner almost indistinguishable from that of cells treated with NGF [58.6 ± 10.3% versus 78.9 ± 6.9% neurite-bearing cells ($P < 0.0001$), with an average neurite length of 90.3 ± 16.4 μm versus 123.2 ± 20.2 μm ($P < 0.0001$), respectively], whereas MTA alone or EGF treatment had almost no effect on neurite outgrowth [2.3 ± 0.7 and 9.1 ± 0.1% of neurite-bearing cells ($P < 0.0001$), with an average neurite length of 5.5 ± 2.3 and 8.4 ± 2.9 μm, respectively] (Fig. 6C and fig. S13). These morphological changes correlated with the abundance of the differentiation markers p21$^{WAF1/CIP1}$ and GAP-43 (Fig. 6C). PC12 cells transfected with the catalytically inactive *PRMT5* mutant of (PRMT5ΔGAGRG) also differentiated in response to EGF [30.5 ± 8.9% of neurite-bearing cells in *PRMT5* mutant–transfected cells versus 2 ± 1.3% in nontransfected cells ($P < 0.001$), with an average length of 60.9 ± 1.7 μm] (Fig. 6D).

As noted, CRAF^{R563K} increased ERK1/2 phosphorylation in HEK293 cells, thereby mimicking the response to growth factors in *PRMT5*-depleted cells (Fig. 4C). We found that PC12 cells expressing the CRAF^{R563K} mutant underwent differentiation when treated with EGF [64.8 ± 14.7% versus 8.2 ± 2.8% neurite-bearing cells ($P < 0.0001$), with an average neurite length of 87 ± 3 μm in EGF-treated CRAF^{R563K}-expressing cells ($P <$ 0,0001)] (Fig. 6E). We also observed this effect in PC12 cells transfected with the BRAF^{R671K} mutant, although this mutant, which has a higher basal kinase activity, increased the number of neurite-bearing cells even in the absence of EGF [87.3 ± 13.4% EGF-treated BRAF^{R671K}-expressing cells versus 30.2 ± 9.2% EGF-treated BRAF^{WT}-transfected cells ($P < 0.0001$), with an average neurite length of 90.5 ± 21.7 μm and 51.1 ± 9.4 μm, respectively] (fig. S12). Moreover, the degree of BRAF methylation induced by NGF versus EGF (fig. S9A) correlated with the activation of PRMT5 activity in response to growth factors (Fig. 2C) and the sustained p-ERK1/2 signal promoted by NGF (Fig. 6A). Together, our data indicate that restriction of ERK1/2 signal amplitude through the methylation of RAF proteins represents a central mechanism in determining the response to growth factors.

DISCUSSION

Activation of the MAPKs ERK1/2 through the RAS signaling pathway plays a crucial role in cell proliferation, differentiation, survival, and oncogenic transformation. Various growth factors that activate this pathway elicit distinct biological responses, with the temporal pattern of ERK1/2 phosphorylation determining which response occurs (*4*, *5*, *36*–*38*). Here, we identify methylation of RAF proteins as a mechanism for modulating RAS-ERK1/2 pathway signaling in response to growth factors. We identify PRMT5 as the arginine methyltransferase involved in limiting ERK1/2 phosphorylation and identify a specific PRMT5 methylation motif (GRG) that is conserved in RAF proteins during evolution. The arginine residue in this motif is involved in the limitation of the kinase activity and the stability of CRAF, thereby contributing to ERK1/2 signal output. We found that inhibiting methylation could redirect growth factor–elicited responses from proliferation to differentiation, indicating that methylation plays an

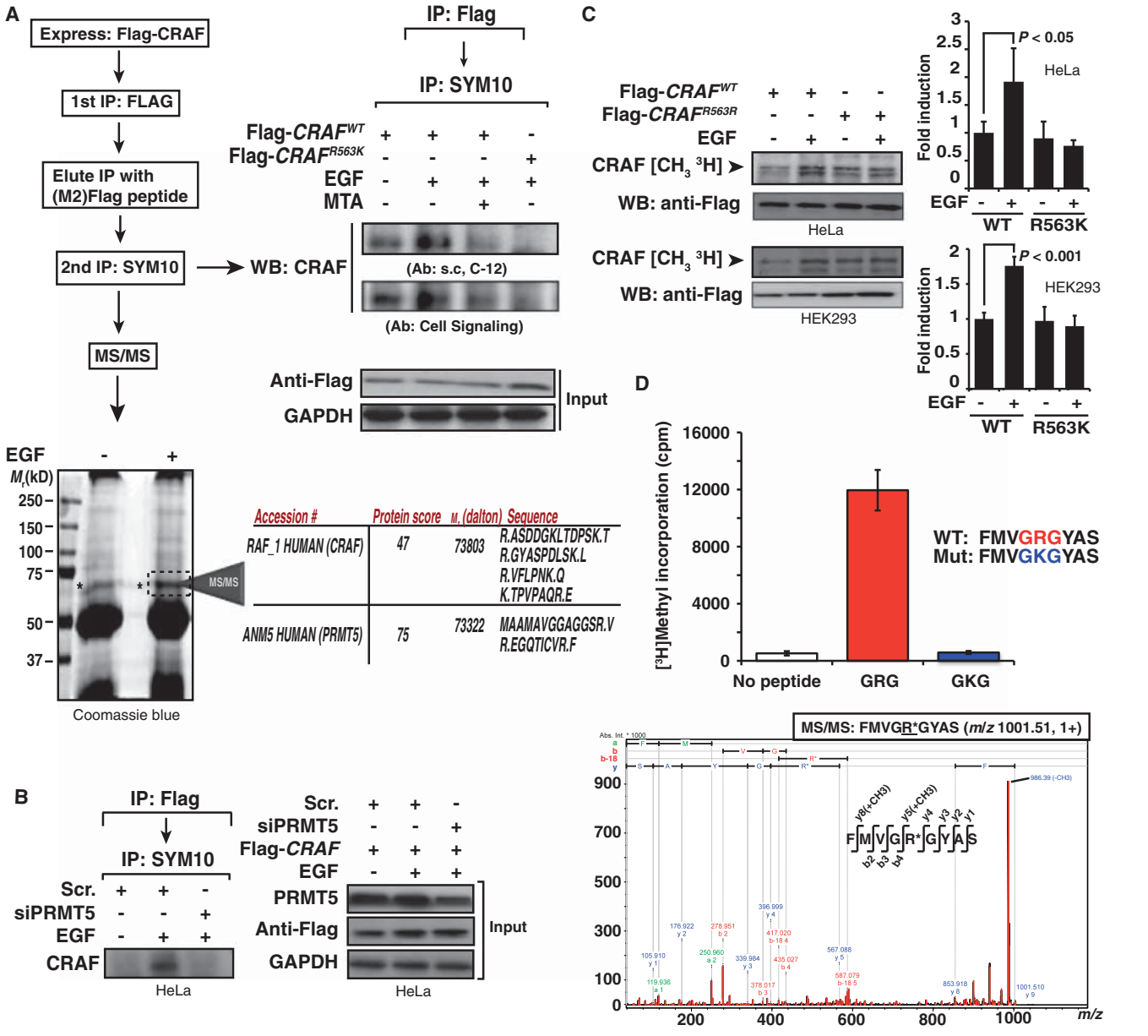

Fig. 5. PRMT5 methylates CRAF on Arg^{563}. (A) HeLa cells were transfected with either Flag-*CRAF*^{WT} or Flag-*CRAF*^{R563K} for 48 hours. Cells were starved and treated with MTA for 2 hours and then treated with EGF for 10 min. Subsequently, Flag-tagged CRAF proteins were immunoprecipitated and the complexes were eluted with M2 peptide. A second immunoprecipitation was performed on the eluates with the anti-sDMA antibody SYM10. The immunocomplexes were analyzed for the presence of CRAF by Western blot and mass spectrometry (MS). Two different antibodies against CRAF were used. Coomassie blue staining of the gel showing the band corresponding to the size of CRAF (*) analyzed by liquid chromatography–mass spectrometry is shown. Table shows the sequences of the identified peptides corresponding to the indicated proteins (lower panel). (B) *PRMT5* depletion inhibits CRAF methylation. HeLa cells transfected with either scrambled or *PRMT5* siRNA for 48 hours and Flag-*CRAF*^{WT} for an extra 24 hours were starved for 2 hours and treated with EGF. Lysates were processed according to the protocol described in (A). Western blot shows the abundance of CRAF. Right panel shows PRMT5 in the initial total lysates. (C) CRAF^{R563K} is not methylated after treatment with growth factors. HeLa or HEK293 cells transfected with either Flag-*CRAF*^{WT} or Flag-*CRAF*^{R563K} were metabolically labeled with [^{3}H]methionine and treated with EGF for 10 min. After immunoprecipitation with the α-Flag antibody, the ^{3}H radioactive signal incorporated in CRAF was assessed autoradiographically after SDS-PAGE. Immunoprecipitated CRAF is shown as loading control. Graph shows quantification of CRAF isoform methylation relative to untreated cells. *P* value was calculated with Student's *t* test (*n* = 3 replicates). (D) CRAF peptide containing GRG motif is methylated in vitro. In vitro methylation assay with immunoprecipitated PRMT5 from EGF-treated Cos-7 cells stably transfected with PRMT5. Assay was performed adding either no peptide, FMVG*R*GYAS peptide, or FMVG*K*GYAS mutant peptide as a control for specificity of arginine methylation. The fragmentation spectrum of the methylated peptide identified by MALDI-TOF/TOF mass spectrometry is shown in the bottom panel. *m*/*z*, mass/charge ratio.

important role in the response to growth factors. The deregulation of this mechanism might be particularly relevant in a number of solid tumors and hematologic malignancies deficient in the methylthioadenosine phosphorylase gene (*MTAP*) (*39*, *40*). MTAP regulates the intracellular amounts of naturally occurring MTA, which accumulates and is secreted from *MTAP*-deficient cells (*41*, *42*).

The ability of methylation to modify the response to growth factors was RAS-ERK1/2 pathway–specific, because PI3K-AKT signaling was not affected. Activation of PI3K by HGF occurs in a RAS-independent manner (*24*), supporting the specificity of the pathway modulation. It is well accepted that, in general, kinases control the amplitudes more than the duration of signals, whereas phosphatases tend to control both (*4*, *5*). Three metrics describe the transient ERK1/2 phosphorylation profile: amplitude, duration, and integrated output (*4*, *5*). Most reactions in the pathway do not affect any of these. RAF proteins are involved in the molecular processes that were identified as important to defining the final amplitude, duration, and integrated response of the signaling output (*4*, *5*). Our data indicate that RAF methylation appears to be a critical posttranslational modification controlling downstream activity, supporting the role of RAF proteins in regulating ERK1/2 phosphorylation profiles. In some cases, the ERK1/2 activation profile also might be determined by the preferential use of a RAF isoform (that is, the preferential signaling of FGF through ARAF) (fig. S8) or by the specific signaling protein complexes used by a particular receptor in a particular cell type.

Whereas lysine methylation is mainly confined to the nucleus, methylation of arginine residues appears to occur throughout the cell (*12*, *13*).

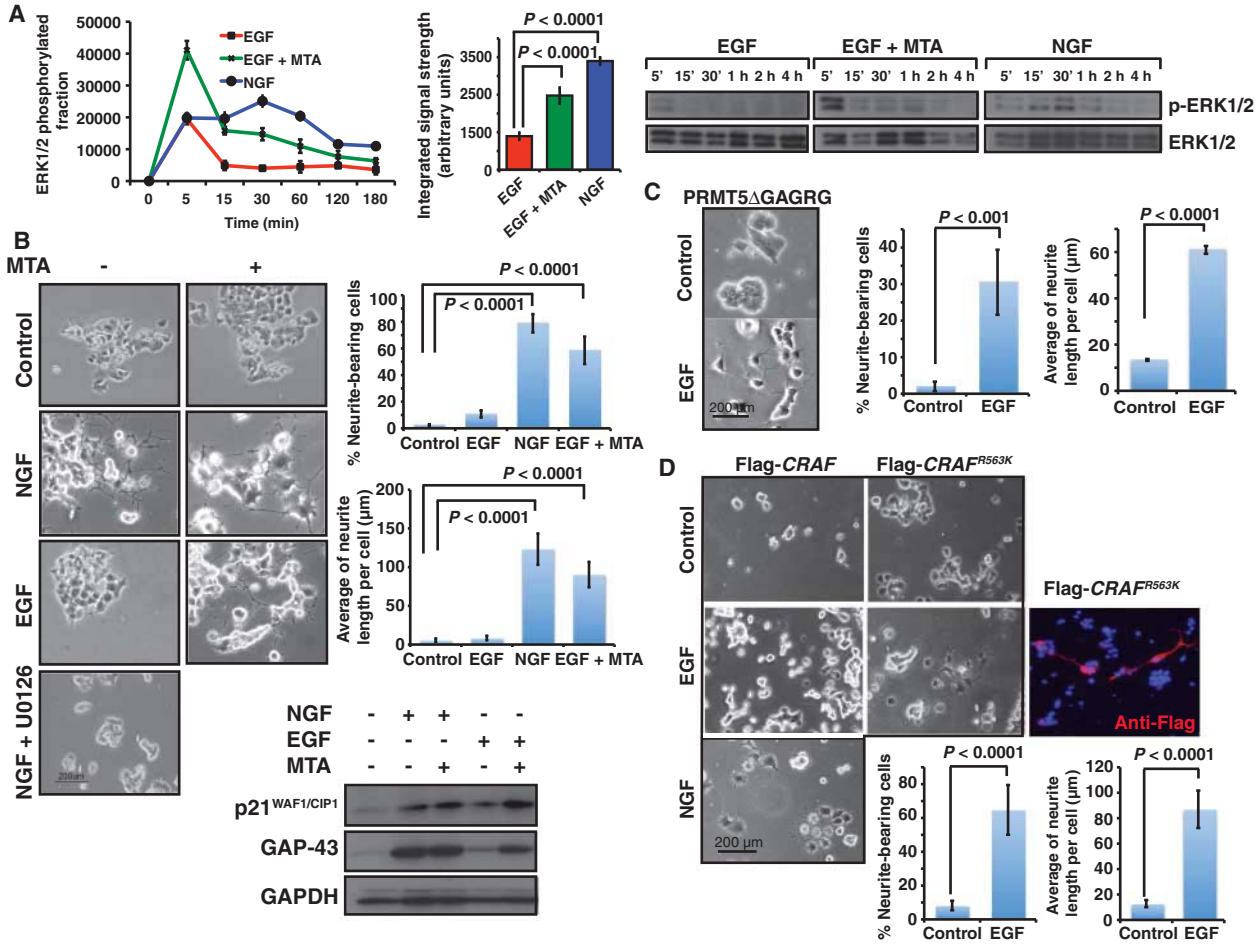

Fig. 6. Modulation of signal amplitude affects the biological response to growth factors. PC12 cells were used as a model for differentiation or proliferation in response to NGF or EGF, respectively. (**A**) Time course of ERK1/2 phosphorylation in response to EGF, EGF + MTA, or NGF. Graph represents the averaged activation profiles of three replicates. (**B**) Treatment of PC12 cells with the combination of EGF and MTA promotes neuronal differentiation. PC12 cells were treated with either MTA, EGF, MTA + EGF, NGF, or NGF + U0126. Representative images of three different experiments after 96 hours of treatment are shown. Neurite outgrowth and length were scored and plotted after 7 days. Data are means ± SD from $n = 3$ experiments. (**C**) Overexpression of catalytically inactive PRMT5ΔGAGRG promotes neuron-like differentiation of PC12 cells in response to EGF. Neurite outgrowth and length were scored and plotted after 4 days. Data are means ± SD from $n = 3$ experiments. (**D**) PC12 cells transiently transfected with Flag-*CRAF*^{R563K} mutant differentiate in response to EGF. Cells transfected with either Flag-*CRAF*^{WT} (1.2 μg) or Flag-*CRAF*^{R563K} (0.2 μg) were treated with EGF or NGF for 96 hours. Representative pictures are shown. Transfected cells were identified with an anti-Flag antibody to detect Flag-CRAF^{R563K} (red staining). DNA appears stained in blue (DAPI). Neurite outgrowth and length in transfected cells were scored and plotted after 4 days. Data are means ± SD from at least 100 transfected cells.

Although PRMT1 is responsible for 85% of methylation reactions (*43*), our results identify PRMT5 as the enzyme involved in restricting ERK1/2 phosphorylation. Thus, PRMT5 limits ERK1/2 signal amplitude in response to growth factors, suggesting that methylation could facilitate the appropriate cellular response to a specific stimulus.

The Shk1 protein kinase, a homolog of *Saccharomyces cerevisiae* Ste20 and mammalian PAKs, is an essential component of a Ras- and Cdc42-dependent signaling cascade required for cell viability, normal morphology, and MAPK-mediated sexual responses in the fission yeast (*23*). Ternary complexes between Skb1 (PRMT5 yeast homolog), Shk1 (PAK yeast homolog), and Cdc42 have been described (*23*), supporting the possible involvement of PRMT5 in the RAS signaling pathway. Indeed, we found that the activity of PRMT5 was regulated by growth factors. RAF activation by RAS is a highly complex process (*10*, *44*). We found that either inhibition of protein methylation or depletion of *PRMT5* enhanced the induction of CRAF's catalytic activity by specific growth factors, identifying a previously unknown level of regulation for RAF proteins. The basal activity of CRAF proteins is not affected by either MTA or *PRMT5* depletion, indicating that modulation of CRAF kinase activity by PRMT5 depends on RAS activation. Thus, we observed that MTA modulates the ERK1/2 signal amplitude in $NRAS^{Q61L}$ mutant cells and not in $BRAF^{V600E}$ mutant cells, which signal independently of RAS (*30*) (fig. S5). In vitro experiments demonstrate that PRMT5 binds to active CRAF and not inactive CRAF. These data confirm the requirement for RAS (a ligand-dependent process) to engage and probably activate PRMT5 in the downstream signaling machinery. The role of protein arginine methylation as posttranslational modification controlling RAS-ERK1/2 signaling pathway is supported by the detection of PRMT5 and CRAF in the same complexes. Indeed, PRMT5 has been described as an HSP90 client, found in association with CRAF or BRAF (*19*), fostering the notion that these molecules can exist within the same protein complex.

PRMT5, the most highly conserved methyltransferase, methylates arginines symmetrically within GRG motifs (*11*). Our results indicate that PRMT5 methylates CRAF at Arg^{563}. In agreement with a role for PRMT5 in limiting ERK1/2 output in RAS pathway signaling, CRAF methylation is increased by exposure to growth factors, and depletion of PRMT5 or mutation of $CRAF^{R563}$ abolishes this effect. Conservative substitution of this arginine with a lysine increased the stability of CRAF and BRAF, suggesting that methylation of this arginine decreases the stability of active RAF molecules. Consistent with this mechanism, the basal activity of $CRAF^{R563K}$ mutant does not differ from that of wild-type CRAF; however, ERK1/2 phosphorylation in response to a growth factor is exaggerated. These data, and similar results obtained with BRAF, suggest that methylation targets active RAF molecules for degradation and that lack of methylation increases the stability of the RAF active molecules, thereby increasing the RAF signal. Consistent with this, we found that CRAF protein was stabilized in the presence of MTA and was highly phosphorylated at Ser^{621}, a modification that has been related to CRAF stabilization (*32*).

We tested the hypothesis that protein methylation is a critical posttranslational modification in growth factor–elicited biological responses with the PC12 model of cellular differentiation (*45*). A particular growth factor will not only initiate the signals that lead to activation of a kinase (thereby affecting signal amplitude) but also activate the mechanisms that deactivate that signal (thereby affecting signal duration). When PRMT5 is inhibited in PC12 cells, the increase in ERK1/2 phosphorylation in response to EGF (signal amplitude) overrides the phosphatase activity triggered by EGF, resulting in a long-lasting signal that reaches the threshold required to elicit neuronal differentiation. Consistent with the proposed mechanism, NGF induced less methylation of BRAF [the major RAF isoform participating in the PC12 differentiation process (*34*)] than did EGF in wild-type cells, but similar to that induced by EGF in *Prmt5*-depleted cells (fig. S9A). $CRAF^{R563K}$ and $BRAF^{R671K}$ mutants also promoted PC12 cell differentiation in response to EGF, supporting the critical role of RAF proteins in controlling the ERK1/2 phosphorylation profile and, consequently, the biological outcome.

In summary, we show that protein arginine methylation limits signal transduction in response to growth factors. We demonstrate that PRMT5 mediates the methylation of RAF, thereby limiting downstream activity in the RAS-MAPK pathway (Fig. 7). This provides a mechanism whereby

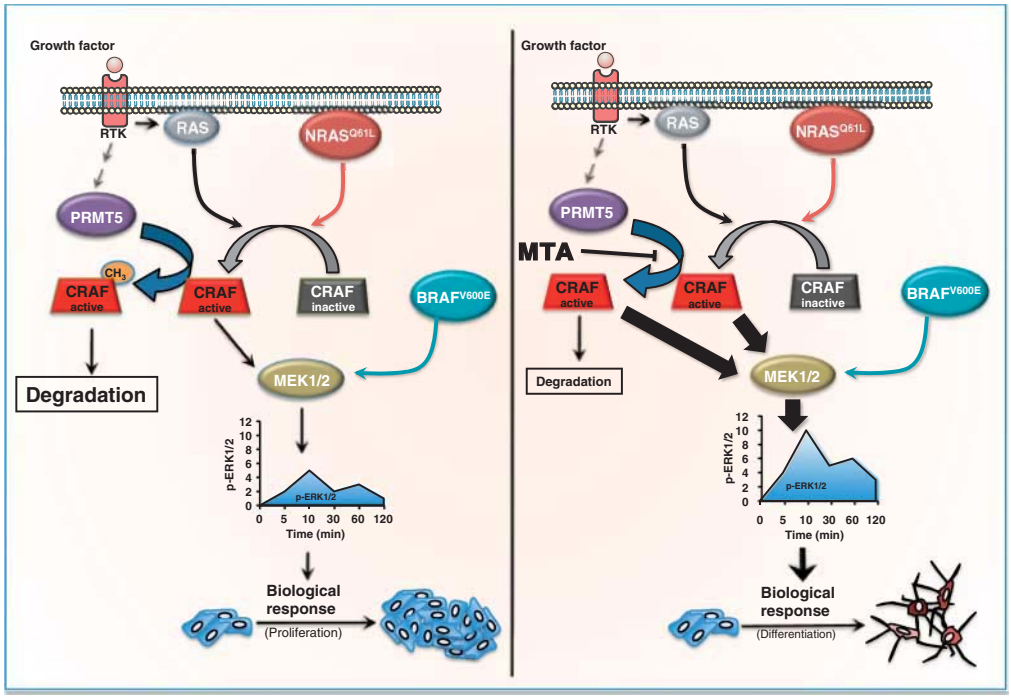

Fig. 7. Model for the limitation of the ERK1/2 signal by PRMT5. Growth factors or mutated RAS activate CRAF and thereby the MAPK signaling pathway. PRMT5 promotes CRAF degradation and limits its catalytic activity, reducing the activation of downstream kinases, such as MEK1/2 and ERK1/2. In contrast, PRMT5 does not affect downstream signals in cells with $BRAF^{V600E}$, which are independent of RAS. Decreasing PRMT5 activity (pharmacologically or by its knockdown) increases the stability of activated CRAF, increasing the amplitude of the ERK1/2 signal and the integrated signal strength and consequently affecting the evoked biological response.

cells can modulate the temporal profile of ERK1/2 phosphorylation. Because cancer cells rely heavily on oncogenic signaling through the RAS-ERK1/2 pathway (*46*), this additional level of regulation may provide additional targets for therapeutic intervention.

MATERIALS AND METHODS

Reagents

c-Met inhibitor PHA-665752 (200 nM) was from Pfizer. MTA, Deaza, c-leucine, cycloheximide (CHX), chloramphenicol, anti-Flag (M2), and anti-Flag (M2) resin were purchased from Sigma-Aldrich. Anti-MKP1 (v-15), anti–lamin A/C (N-18), anti-PRMT5 (C-20), anti-PRMT1 (B-2), c-Met, anti-CRAF (C-12), anti–GAP-43 (B-5), and anti-ERK2 (C-14) were purchased from Santa Cruz Biotechnology. Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase), used to demonstrate equal loading in Western blots, was from Trevigen. Anti–MAPK p-ERK1/2, anti–p-MEK1/2, anti–p-AKT, anti-ERK1/2, anti-MEK1/2, anti-AKT, anti–p-c-Met ($Tyr^{1234/1235}$), and U0126 were from Cell Signaling. Anti-RAS anti-$p21^{WAF1/CIP1}$ and anti-Sam68 were from BD Biosciences, Pharmingen. L-[*methyl*-^{3}H]Methionine (70 to 85 Ci/mmol), *S*-adenosyl-L-[*methyl*-^{3}H]methionine, and [^{35}S]methionine were obtained from GE Healthcare.

Constructs

pcDNA3.1-Flag-*CRAF* was provided by A. Shaw (Washington University, St. Louis, Missouri). pEF_2-Flag-*PRMT5* and pEF_2-*PRMT5*Δ*GAGRG* mutant expression vectors were a gift from S. Petska (R. W. Johnson Medical School, New Brunswick, New Jersey). pcDNA3.1-Flag-$CRAF^{R563K}$ and pLNCX2-*myc*-$BRAF^{R671K}$ mutants were generated with a QuikChange Site-Directed Mutagenesis kit from Stratagene.

Cell culture, transfection, and growth factor stimulation

The 37-31E and 37-31T mouse melanoma cell lines have been previously described (*47*). B16F1, HEK293, PC12, U87, A375, and Cos-7 cells were obtained from the American Type Culture Collection. B16F1, HEK293, A375, and U87 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen) and penicillin/streptomycin (P/S) [P (100 U/ml)/S (100 μg/ml)] (Invitrogen). Cos-7 cells were grown in RPMI 1640 (Invitrogen) plus 10% FBS and P/S. PC12 cells were maintained in F12K medium (Invitrogen) containing 10% horse serum (Invitrogen), 5% FBS, and P/S. SKMel147 human melanoma cells were a gift from M. S. Soengas (Centro Nacional de Investigaciones Oncológicas, Madrid, Spain). UACC903 human melanoma cells were a gift from P. Pollock (TGen). Wild-type MEFs were provided by M. Baccarini (Max F. Perutz Laboratories, Austria). For growth factor treatments, cells were starved for 3 hours or overnight depending on cell requirements. MTA (0.4 mM) and Deaza (20 mM) were added 3 hours before addition of growth factors. c-Leucine (20 mM) was added overnight before growth factor treatment. Cells were stimulated with HGF (40 ng/ml), FGF1 (40 ng/ml), and FGF2 (40 ng/ml) (R&D Systems), EGF (50 ng/ml) (Millipore), insulin (100 nM), MSH-α (100 nM), NGF, or TPA (200 nM) (Sigma-Aldrich) and harvested at the times indicated. Constructs were transfected into Cos-7, PC12, B16F1, or HEK293 cells with Lipofectamine (Invitrogen), following the manufacturer's recommended protocol.

Isolation of primary rat hepatocytes

Primary rat hepatocytes were isolated as previously described (*48*). Cells were plated onto collagen-coated six-well dishes (type I collagen from rat tail; Collaborative Biomedical) at 10^6 cells per dish. Cultures were maintained in minimal essential medium supplemented with 5% FBS, non-essential amino acids, 2 mM glutamine, and antibiotics (Invitrogen). After a 6-hour incubation, the culture medium was removed and replaced with the same medium without FBS. Where indicated, cells were pretreated with MTA for 3 hours and then exposed to HGF for various periods of time.

Western blot and immunoprecipitation

Cells were lysed with M-PER (Pierce) in the presence of protease inhibitor cocktail (Roche). Fifty micrograms of protein was separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) using 4 to 20% or 12% gels (Invitrogen). Proteins were transferred to polyvinylidene difluoride membranes and probed with primary antibodies. Proteins were visualized with secondary antibodies coupled to horseradish peroxidase enhanced chemiluminescence (GE Healthcare) or SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Immunoprecipitations were performed in M-PER lysis buffer (Pierce) after the addition of Protein A/G Agarose PLUS (Santa Cruz Biotechnology) or anti–Flag resin (Sigma-Aldrich). Samples were fractionated and visualized as described above. After stripping with 5 mM tris-HCl (pH 2), membranes were blocked and incubated with other antibodies as indicated.

Quantification of p-ERK1/2 (fold induction with respect to the untreated cells) in Fig. 4C and fig. S10A was performed by dividing the densitometric value obtained for p-ERK1/2 by the densitometric value obtained from all the proteins (Ponceau S staining) from the same lane. Then, the resulting number was divided by the number obtained in the untreated cells transfected with the wild-type isoform.

Immunofluorescence

Cells were grown on glass coverslips. Briefly, after treatment, cells were washed in cold phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS for 10 min, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were blocked with PBS containing 3% bovine serum albumin (BSA) and then incubated with the primary antibody overnight at 4°C. Proteins were visualized by confocal microscopy (Espectral FV1000, Olympus) with fluorescein isothiocyanate– or phycoerythrin-conjugated secondary antibodies. DAPI (4′,6-diamidino-2-phenylindole) was used for nuclear staining.

RAF kinase assays

Coupled RAF kinase assays were performed with immunoprecipitated ARAF, BRAF, or CRAF as previously described (*49*). Briefly, immunoprecipitated CRAF was incubated with 20 μl of MKK buffer [30 mM tris (pH 7.5), 0.1 mM EDTA, 10 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 5 mM NaF, 0.2 mM Na_3VO_4, 0.3% (v/v) β-mercaptoethanol, GST-MKK (6.5 μg/ml), GST-ERK (100 μg/ml), and 0.8 mM adenosine 5′-triphosphate (ATP)] and incubated at 30°C for 10 to 30 min. Reactions were terminated by the addition of 20 μl of KILL buffer [30 mM tris (pH 7.5), 6 mM EDTA, 0.1% (v/v) Triton X-100, 5 mM NaF, 0.2 mM Na_3VO_4, and 0.3% (v/v) β-mercaptoethanol]. Five microliters of reaction supernatant was incubated in triplicate with 25 μl of myelin basic protein (MBP) buffer [50 mM tris (pH 7.5), 0.1 mM EDTA, 10 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 5 mM NaF, 0.2 mM Na_3VO_4, 0.1 mM ATP, 0.3% β-mercaptoethanol, BSA (200 μg/ml), MBP (1 mg/ml), 50 μCi [γ-^{32}P]ATP (5000 Ci/mmol)] for 10 min before blotting onto P81 paper and placing in a 25 mM orthophosphate solution. P81 paper was washed extensively and the amount of [γ-^{32}P]ATP incorporated into MBP was measured by Cherkenov counting. All experiments were performed in triplicate. ERK kinase activity was measured with MBP as a substrate. When MEK was used as the unique CRAF substrate, the kinase assays were resolved by SDS-PAGE and Western blots were performed against p-MEK1/2 and MEK1/2. All experiments were performed at least in triplicate.

Nuclear extracts
Nuclear extracts were obtained with an N-PER kit (Pierce) following the manufacturer's instructions.

Pulse chase
Nontransfected and PRMT5 siRNA–transfected HeLa cells were incubated for 30 min in a CO_2 incubator in 5 ml of DMEM without Met and Cys. Then, 2 ml of fresh DMEM without Met and Cys containing 10% dialyzed FBS and [^{35}S]methionine (250 μCi/ml) were added to each dish and incubated for 30 min. Cells were washed once with warm PBS, incubated with complete DMEM, and then chased for 24 hours. In experiments performed with CHX, cells were treated with CHX (5 μg/ml) and chased at the times indicated. Immunoprecipitation of the indicated proteins was performed with 500 μg of total protein.

In vivo methylation assays
In vivo methylation assays were performed as described (*50*, *51*). Briefly, cells were incubated with CHX (100 μg/ml) and chloramphenicol (40 g/ml) in complete medium for 30 min. The medium was then replaced with DMEM without methionine, supplemented with penicillin, streptomycin, and 10% fetal calf serum containing L-[*methyl*-^{3}H]methionine (10 μCi/ml), and the methyl donor and cells were incubated for an additional 3 hours in the presence of the same protein synthesis inhibitors. The cells were lysed in 10 mM tris-HCl–100 mM NaCl–2.5 mM $MgCl_2$ (pH 7.4) containing 0.5% Triton X-100, 1% aprotinin, leupeptin (2 μg/ml), pepstatin A (2 μg/ml), and 0.1 mM dithiothreitol (DTT), and proteins of interest were immunoprecipitated with the appropriate antibodies. For experiments with PC12 cells, siRNA transfection was performed 50 to 60 hours before the experiment. For experiments with HeLa or HEK293 cells, complementary DNA (cDNA) constructs and siRNAs were transfected 24 hours before metabolic labeling.

In vitro methylation assays
Flag-PRMT5 was expressed in HeLa cells and immunopurified with Flag resin (Sigma-Aldrich). Enzymes immobilized in the resin were then incubated with either 4 μg of MBP (Sigma-Aldrich), 100 μg of unmodified CRAF peptide (FMVGRGYAS), or 100 μg of modified CRAF peptide (FMVGKGYAS) (GeneScript Inc.) in the presence of 10 μCi of *S*-adenosyl-L-[*methyl*-^{3}H]methionine for 1 hour at 30°C in a final volume of 50 μl of methylation buffer [100 mM tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM DTT]. Peptides were purified with ZipTip C18 Pipette Tips (Millipore). Five micrograms of peptide was loaded onto P81 papers, washed, and counted by liquid scintillation. Ten micrograms of peptide was analyzed by matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF)/TOF mass spectrometry on an Autoflex instrument (Bruker). When MBP was used as substrate, the whole reaction was resolved by SDS-PAGE. Then, the ^{3}H signal was captured by exposing the gels to a film.

In vitro binding assays
Recombinant PRMT5 (2 μg; Novus Biologicals) and recombinant full-length inactive GST-CRAF (2 μg; Sigma-Aldrich) or active GST-CRAF (N-terminal GST-tagged, residues 306 to end) (2 μg; GeneScript Inc.) were incubated for 1 hour at 30°C in a final volume of 50 μl of methylation buffer [100 mM tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM DTT]. Then, PRMT5 was immunoprecipitated and CRAF was detected by immunoblotting.

Phosphatase activity assays
The MAPK phosphatases 1 and 2 (MPK1/2) were immunoprecipitated from nuclear extracts, and phosphatase activity was measured with Ser/Thr Phosphatase Assay Kit 1 (K-R-pT-I-R-R) (Millipore) according to the manufacturer's protocol.

siRNA transfection
PRMT1 (*52*), *PRMT5* (*53*), *CRAF* (*30*), and scrambled siRNA were purchased from Dharmacon. Cells were transfected with siRNAs (100 nM final concentration) using Lipofectamine 2000. The assays were performed 48 to 72 hours after transfection.

PC12 cell differentiation assay
The assay was performed as previously described (*54*). Briefly, PC12 cells were treated with EGF, NGF, EGF plus MTA, or NGF plus U0126 for 7 days. Cells with neurites >20 μm in length were considered neurite-bearing. At least 30 fields measured at 20× magnification were counted per condition. Average neurite length was calculated with ImageJ64 software where at least 100 cells from each experimental condition were scored from randomly chosen fields.

Liquid chromatography–mass spectrometry analysis
Samples of interest were separated on a 10% SDS-PAGE gel, and the gel was stained with colloidal Coomassie blue. Protein bands of interest were cut from the gel and digested with modified porcine trypsin (Trypsin Gold, Promega). Digests were analyzed on an Esquire HCT IT mass spectrometer (Bruker) coupled to a nano-HPLC (high-performance liquid chromatography) system (Ultimate, LC Packings) as described (*55*).

Statistics
All quantitative results are presented as the means ± SD of independent experiments. Statistical differences between two groups of data were analyzed by Student's *t* test. After testing the nonparametric distribution of PC12 neurite-outgrowth quantification data, we performed a Mann-Whitney test to analyze the statistical differences among the different groups.

SUPPLEMENTARY MATERIALS
www.sciencesignaling.org/cgi/content/full/4/190/ra58/DC1
Fig. S1. MTA increases ERK1/2 phosphorylation in response to HGF in primary rat hepatocytes.
Fig. S2. Modulation of HGF induced MEK1/2-ERK1/2 signaling by MTA in melanoma cells.
Fig. S3. MTA modulates ERK1/2 phosphorylation upon specific growth factor treatment in human cells, and protein methylation inhibition modulates ERK1/2 signal amplitude in response to HGF.
Fig. S4. PRMT1, PRMT3, and PRMT8 are not responsible for the MTA-mediated effect.
Fig. S5. MTA signal amplitude modulation of ERK1/2 is RAS activation–dependent.
Fig. S6. CRAF and BRAF immunoprecipitate and colocalize with PRMT5 in the cytoplasm.
Fig. S7. MTA regulates growth factor–induced BRAF kinase activity.
Fig. S8. Differential activation of RAF isoforms by growth factors.
Fig. S9 MTA treatment inhibits CRAF degradation in SKMel147 cells.
Fig. S10. $BRAF^{R671K}$ mutant shows the same response as $CRAF^{R563K}$. ARAF stability is not regulated by MTA in SKMel147 cells.
Fig. S11. A small fraction of total CRAF is recruited to the membrane in response to growth factor signaling. Detection by MALDI of both the unmethylated and the methylated CRAF peptides.
Fig. S12. BRAF is in vivo methylated. $BRAF^{R671K}$ mutant mimics the MTA-induced effects on PC12 cells.
Fig. S13. PC12 differentiation assay.
Methods
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2. Methylthioadenosine (MTA) inhibits melanoma cell proliferation and *in vivo* tumor growth.

Metiltioadenosina (MTA) inhibe la proliferación en células de melanoma y el crecimiento tumoral *in vivo*.

El melanoma es la forma más mortal de cáncer de piel para la cual no existe un tratamiento efectivo. El MTA (metiltioadenosina) es un nucleósido que presenta diferentes efectos en células normales y transformadas. Puesto que varias investigaciones han demostrado que MTA es capaz de promover respuestas anti proliferativas y pro apoptóticas en ciertos tipos celulares, hemos intentado averiguar si MTA puede tener cierto potencial terapéutico en el tratamiento del melanoma. Para averiguarlo hemos realizado ensayos de proliferación celular y viabilidad usando seis líneas celulares de melanoma (humanas y de ratón) que presentan diversas mutaciones en la vía de RAS. También hemos probado las capacidades terapéuticas de MTA en un modelo de ratón *in vivo* y hemos investigado las capacidades anti proliferativas y pro apoptóticas del MTA. Nuestros resultados muestran que el tratamiento con MTA es capaz de inhibir la proliferación celular y la viabilidad en las líneas de melanoma que usamos. Además, MTA fue capaz de inhibir efectivamente el crecimiento tumoral *in vivo*. El análisis molecular de los tumores y los resultados obtenidos *in vitro* indican también que los efectos de MTA son citostáticos en lugar de pro apoptóticos ya que el uso de MTA inhibe la fosforilación de AKT y de la proteína ribosomal S6, desregulando la ciclina D1. En resumen, nuestros datos muestran que MTA es capaz de inhibir la proliferación celular *in vitro* y el crecimiento

tumoral *in vivo*, presentando a MTA como una droga potencialmente efectiva en el tratamiento del melanoma.

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Methylthioadenosine (MTA) inhibits melanoma cell proliferation and *in vivo* tumor growth

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Abstract

Background: Melanoma is the most deadly form of skin cancer without effective treatment. Methylthioadenosine (MTA) is a naturally occurring nucleoside with differential effects on normal and transformed cells. MTA has been widely demonstrated to promote anti-proliferative and pro-apoptotic responses in different cell types. In this study we have assessed the therapeutic potential of MTA in melanoma treatment.

Methods: To investigate the therapeutic potential of MTA we performed *in vitro* proliferation and viability assays using six different mouse and human melanoma cell lines wild type for RAS and BRAF or harboring different mutations in RAS pathway. We also have tested its therapeutic capabilities *in vivo* in a xenograft mouse melanoma model and using variety of molecular techniques and tissue culture we investigated its anti-proliferative and pro-apoptotic properties.

Results: *In vitro* experiments showed that MTA treatment inhibited melanoma cell proliferation and viability in a dose dependent manner, where BRAF mutant melanoma cell lines appear to be more sensitive. Importantly, MTA was effective inhibiting *in vivo* tumor growth. The molecular analysis of tumor samples and *in vitro* experiments indicated that MTA induces cytostatic rather than pro-apoptotic effects inhibiting the phosphorylation of Akt and S6 ribosomal protein and inducing the down-regulation of cyclin D1.

Conclusions: MTA inhibits melanoma cell proliferation and *in vivo* tumor growth particularly in BRAF mutant melanoma cells. These data reveal a naturally occurring drug potentially useful for melanoma treatment.

Background

Melanoma is a common skin cancer resulting in high morbidity and mortality. The development of effective therapeutics designed to target melanoma has become the recent focus of research to improve the melanoma patient's prognosis.

In mammalian cells, 5'-Methylthio-5'deoxyadenosine (MTA) is formed from decarboxylated S-adenosylmethionine in the biosynthesis of spermidine and spermine, and is cleaved by MTA phosphorylase (MTAP) into adenine and 5'-methylthio-5'deoxyribose-1-phospate, which are used for the salvage of ATP and methionine respectively [1].

The *MTAP* gene lies on 9p21, close to the gene *CDKN2A* that encodes the tumor suppressor proteins $p16^{INK4A}$ and $p14^{ARF}$ being widely expressed in normal cells and tissues [2]. The *INK4A-ARF* locus on chromosome 9p21, (encoding $p16^{INK4a}$ and $p14^{ARF}$), is often deleted in human melanoma [3]. Interestingly, *MTAP* and *CDKN2A* are frequently homozygously co-deleted otherwise, inactivated in tumor cells including melanoma [4], resulting in higher intra and extracellular MTA levels [5]. A wide variety of biological responses to MTA have been reported both *in vivo* and in cell culture. While physiological intracellular concentrations of MTA in the nM range might have a tumor-supporting role in MTAP deficient-melanoma cells [5], the administration of higher concentrations of MTA (μM) interfere with cell proliferation, lymphocyte activation, tumor development, invasiveness and the regulation of apoptosis [6-10]. Moreover, it has been shown that MTA has a differential effect in normal and transformed cells. While hepatocarcinoma cells undergo apoptosis when treated with MTA, normal hepatocytes and normal

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human fibroblast remain viable and are protected from okadaic acid-induced programmed cell death [5,10,11]. Importantly, MTA has been tested in mice and rats and found to be non-toxic at high doses even when given over extended periods [12,13].

Although the mechanisms of action of MTA are not fully understood, it has been postulated that the inhibition of polyamine synthesis could be responsible for the cytostatic effects of MTA. Moreover, MTA has been shown to interfere with key cell signaling pathways, being able to inhibit growth-factor induced protein tyrosine phosphorylation and to increase intracellular cAMP levels through the inhibition of cAMP phos-phodiesterase [14,15]. Additionally, MTA inhibits protein methylation, modulating cell signaling and protein expression [9,16,17].

A number of studies have demonstrated the effects of MTA in tumoral cell lines. However, *in vivo* studies have been restricted to gastroenterological tumor models or brain autoimmune disease. Besides the continuous efforts from the scientific community, there are not effective therapeutic approaches for melanoma treatment. In this study we explored the therapeutic properties of MTA in melanoma treatment. We used several human and mouse melanoma cell lines having different mutational status respect RAS, and BRAF proteins, and investigated the inhibition capabilities of MTA *in vitro*. We also performed *in vivo* studies using a mouse melanoma xenograft model showing the effectiveness of MTA in melanoma treatment.

Materials and methods

Cell lines

37-31E mouse melanoma cells were described previously [18-20]. UACC903 cells were a gift from J. Trent (P. Pollock, Tgen, Phoenix, AR, USA). SkMel147 and SKMel103 cells were obtained from M. Soengas (CNIO Madrid, Spain) and MeWo and SKMel28 cells were purchased from the ATCC. 37-31E, SkMel147, SKMel103 and MeWo cells were maintained in DMEM with 10% FBS, penicillin/streptomycin. 37-31E cells were supplemented with EGF (50 ng/ml) (Invitrogen, Carlsbad, CA, USA) and Insulin (4 μg/ml) (Invitrogen, Carlsbad, CA, USA) and grown at 37°C and 5% CO_2 conditions. UACC903 were maintained in RPMI medium with 10% FBS, penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA).

Antibodies and Western Blot analysis

Cells were lysed in RIPA buffer containing phosphatase and protease inhibitors (Sigma-Aldrich, Saint Louis, MO, USA). Liquid nitrogen frozen tumor samples were homogenized in RIPA buffer. 50 μg of total protein lysates were separated by SDS-PAGE and transferred to a membrane. After blocking, membranes were blotted against different primary antibodies and developed using horseradish peroxidase linked secondary antibodies and ECL (GE Healthcare, Barcelona, Spain). Cyclin D1 antibody was from Santa Cruz; phospho-Erk1/2 (Thr202/Tyr204), cleaved-caspase-3, p-S6 (Ser235/236), and phospho-Akt (Thr308) antibodies were from Cell Signaling (Danvers, MA USA); p-Bad was from Genscript, Piscataway, NJ, USA, Ki67 was from Master diagnostica, (Granada, Spain) and GAPDH was from Trevigen (Gaitherburg, MD, USA). CD31 antibody was from DAKO (Spremberg, Denmark). The anti-MTAP antibody was the generous gift of Dr. D.A. Carson (University of California San Diego, CA, USA), and was used at a 1:1.000 dilution.

Proliferation assays

Cells were seeded one day before treatment (50,000 cells per well (37-31E, MeWo, SkMel103, SkMel147, UACC903,) or 75,000 cells per well (Colo829)). Time point treatments were done in triplicates. Number of viable cells at different time points was analyzed by using Guava-Viacount reagent (Guava Technologies, Hayward, CA, USA), in a cell counter (Viacount; Guava Technologies, Hayward, CA, USA).

Cell cycle analysis

Cells were grown in complete media and treated for 48 h with 10 μM of MTA. Time point treatments were done in triplicates. Then, medium and cells were recollected and after centrifugation, cells were fixed and stained with the Cell Cycle Analysis Guava-Viacount reagent (Guava Technologies, Hayward, CA, USA). Samples were analyzed with the Guava cytometer PCA (Guava Technologies Hayward, CA, USA).

In vivo studies

Five to six month old male FVB/N mice were injected subcutaneously with one million cells in PBS. When tumors reached between 50-100 mm^3, mice were treated with either DMSO or MTA (96 μmol/kg body weight), a dose selected for its previously shown efficacy and lack of toxicity in other *in vivo* models [13,21]. MTA was prepared from S-adenosylmethionine (Europharma, Madrid, Spain) as described elsewhere [22]. Treatments were done by IP injection daily. Control mice were treated with the same volume of DMSO (100 μl). Tumor size and mice weight was monitored every two days. Tumor volume was calculated with the equation $(d^2*D)*(\pi/6)$ (d = small-diameter; D = big-diameter). When mice were sacrificed, tumors were dissected and processed. All the animal procedures have been approved and supervised by the animal care and ethical committee of the Vall d'Hebron Research Institute.

Immunohistochemistry, Immunofluorescence, TUNEL assay and microvessel density quantification

Paraffin-embedded tumor samples were subjected to immunocytochemistry according to the manufacturer's antibody protocol. Samples were developed either by using secondary antibodies linked to horseradish peroxidase (HRP) and diaminobenzidine (DAB) as a substrate or by immunofluorescence. Tumor samples were used to perform a TUNEL assay as described previously [23]. Apoptosis and proliferating cells were quantified by calculating the average of positive cells in ten fields (10×). For microvessel quantification, tumor samples were stained for CD31. Then, the integrated density of CD31 fluorescence per field (20× magnification = 587590 μm^2) was measured using ImageJ software (NIH). Ten fields per sample were quantified for a total of 5 DMSO treated tumor samples and 5 MTA treated tumor samples.

Colony formation assays

Three hundred cells were seeded. Treatments were added next day. Plates were incubated at 37°C and 5% CO_2 until differences between the treatment conditions were noticeable. Media was changed every 2 days. Plates were washed with PBS, fixed with 4% formaldehyde (Sigma-Aldrich, Saint Louis, MO, USA) in PBS for 10 minutes, and stained with crystal violet. Finally, representative pictures were taken and the number of clones was quantified. At least two biological replicates with three technical replicates each were performed for every cell line.

Motility assays

For motility assays 5×10^4 cells were seeded in a 24 multi-well plate trans-wells (Corning). Following treatment cells were washed with 1× PBS and fixed with 1% glutaraldehyde in PBS. Cells were then stained with an aqueous solution of 0.1% crystal violet. After destaining in water, non-migrating cells in the top of the trans-well were removed, and stained migrating cells in the bottom of the trans-well were destaining with PBS containing 0.2% Triton X-100. The O.D. was then measured at 590 nm[24].

mRNA samples and qRT-PCR

Fresh tumor tissues were disrupted using a rotor-stator homogenizer. mRNAs from tumors and cell lines were purified using RNeasy Kit (Quiagen). Amount and quality of RNA was assessed by spectrometrical measurements. Two hundred ng of RNA per sample were used to obtain cDNA using SuperScript™ III First-Strand Synthesis System for RT-PCR following the manufacturer's recommendations (Invitrogen, Carlsbad, CA USA). qRT-PCR was performed using validated Taqman Probes (VEGF: Mm 01281447_m1, and 18 S RNA: Hs 03003631_g1); (Applied Biosystems, Foster City, CA USA). qRT-PCR was performed according to manufacturers recommendations in a SDS 7900HT System. 18 S RNA was used as an internal control. Results were calculated using ΔΔCt method.

Statistics

Comparisons protein expression and tumor size among cell lines or treatment groups were done by two-sided *t* test (Microsoft Excel, Microsoft, Redmond, WA, USA). Clonogenic assays were analyzed using Wilcoxon Signed-Rank Test [25](Vassar Stats, Poughkeepsie, NY USA).

Results

Methylthioadenosine (MTA) inhibits melanoma cell proliferation

To investigate the inhibition capabilities of methylthioadenosine (MTA) on melanoma cells, we performed cell proliferation assays. Melanoma cell lines harboring wild type NRAS and BRAF (37-31E and MeWo), $NRAS^{Q61L}$ mutation (SKMel 103 and SKMel 147) or $BRAF^{V600E}$ mutation (UACC903, Colo 829) were grown in complete medium and in the presence of increasing concentrations of MTA. Wild type NRAS and BRAF melanoma cells (37-31E, MeWo) (Fig 1A), and melanoma cells harboring $NRAS^{Q61L}$ (SKMel 147 and SKMel 103) (Fig 1B) showed a 50% inhibition of cell proliferation at 9.8 ± 0.4 μM, 18.9 ± 1.2 μM, 10.01 ± 0.2 μM and 21.2 ± 0.2 μM respectively, while the GI50 for $BRAF^{V600E}$ mutant cell lines was 6.1 ± 0.4 for UACC903 cells and 8.2 ± 0.3 for Colo 829 cells (Fig 1C). $BRAF^{V600E}$ mutant cells showed a significant lower proliferation rate ($p < 0.05$) at 96 h using 10 μM MTA compared with wild type or $NRAS^{Q61L}$ mutant cells (Fig 1D). All melanoma cell lines were significantly ($p < 0.01$) more sensitive than normal MEFs (GI50>600 μM) (additional file 1, panel A), reaching the maximum inhibition of proliferation between 10 μM and 600 μM concentration of MTA depending on the cell line (Fig 1). Moreover, cells harboring deletions in p16INK4a showed lower levels of MTAP, and 3 out of 4 of these cell lines appeared to be more sensitive to MTA treatment (Fig 1E).

These results indicate that MTA is effective inhibiting *in vitro* melanoma cells proliferation. Interestingly, the results also suggest that BRAF mutant cells and probably cells showing low levels of MTAP would be more sensitive to the MTA treatment.

MTA reduces melanoma cells survival

We next investigated whether MTA interferes with melanoma cells survival capabilities. To that end, we performed clonogenic assays in several melanoma cell lines adding increasing concentrations of MTA (1 μM

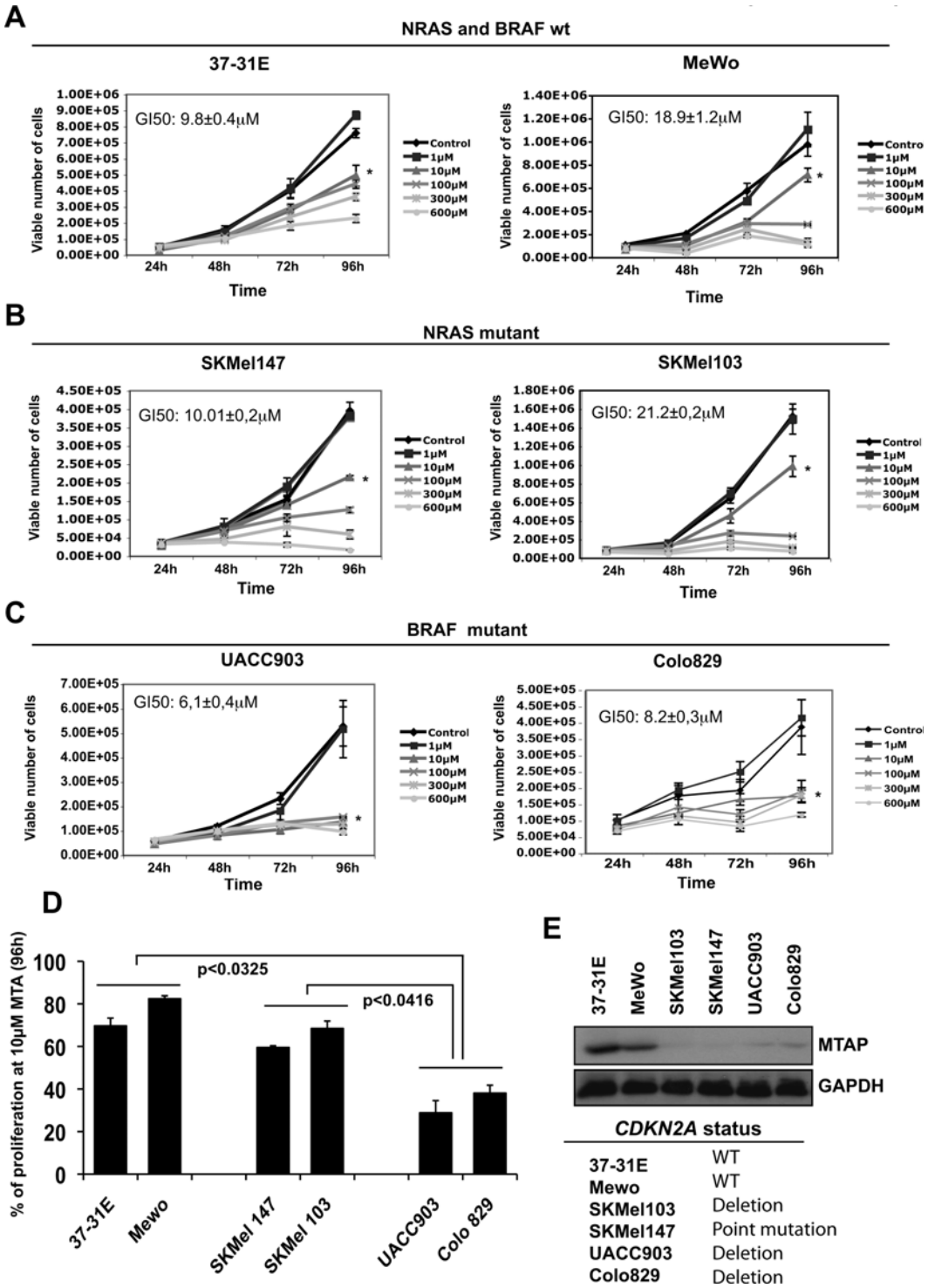


Figure 1 Methylthioadenosine inhibits melanoma cell growth. Proliferation assays of (A) 37-31E and MeWo (wild type RAS BRAF), (B) SKMel147 and SKMel103 ($NRAS^{Q61L}$ mutated) and (C) UACC903 and Colo829 ($BRAF^{V600E}$ mutated) melanoma cells in complete medium with increasing concentrations of Methylthioadenosine (MTA). Medium was changed adding fresh MTA every 48 h. Proliferation assays were performed by counting the number of viable cells using Guava-Viacount reagent (Guava Technologies) in a cell counter (Viacount) at the time points indicated. Asterisk indicate $p < 0.05$. (D) Graph showing the percentage of cell proliferation inhibition at 96 h treated with 10 μM MTA. *p*-values were calculated performing a *t*-student test. (E) Western-blot showing the levels of MTAP in the different cell lines. The mutational status of *CDK2NA* for the different cell lines is showed in the table.

to 1 mM). Interestingly, 1 μM concentration of MTA promoted an increase in the number of colonies in 37-31E cells, with no detectable effects in the other of cell lines tested except for the $BRAF^{V600E}$ mutant cell line Colo829 where 1 μM of MTA totally suppressed the colony formation. At 10 μM of MTA $BRAF^{V600E}$ mutant cell lines were significantly more sensitive ($p < 0.02$) than RAS^{Q61L} mutant cell lines. The addition of 100 μM of MTA, or higher concentrations, was very effective inhibiting melanoma cell viability, independently of the RAS pathway mutational status (Fig 2). Altogether, the results showed that at 100 μM or higher concentrations of MTA there was not any difference in viability in response to MTA treatment among cell types harboring wild type or mutant RAS pathway. However, at lower concentrations (10 μM) $BRAF^{V600E}$ mutant cells were slightly more sensitive than wild type or RAS^{Q61L} mutant cell lines.

MTA treatment inhibits melanoma *in vivo* tumor growth

In view of to the inhibitory capabilities of MTA *in vitro*, we tested whether or not MTA was effective inhibiting *in vivo* tumor growth. To answer this question we performed an *in vivo* experiment in a melanoma xenograft mouse model. The FVB/N syngenic 37-31E melanoma cell line was subcutaneously injected into FVB/N mice. Then, mice were divided in two groups and treated with DMSO (control) and MTA respectively. When tumors reached between 50-100 mm^3 the drug was administrated daily via intraperitoneal letting the tumors grow for a total of thirty days. Mice treated with MTA showed a significant decrease (47%) in the tumor volume ($p < 0.001$) compared with the controls (DMSO) (Fig 3A) with no evident toxic effects according to the mice weight (data not shown) and liver function tests ($ALT_{(iu/L)}$: control: 19 ± 2; MTA: 18 ± 3; $AST_{(iu/L)}$: control: 45 ± 4; MTA: 54 ± 3).

We investigated the status of p-Erk1/2, p-Akt and p-S6 by immunohistochemistry using paraffin-embedded tumor samples. Tumor samples treated with MTA showed a diminished activity of the PI3K and mTOR pathways according to the p-Erk1/2, p-Akt and p-S6 levels (Fig 3B).

Altogether, these results indicate that MTA treatment is effective blocking melanoma *in vivo* tumor growth interfering with the activation of PI3K and mTOR pathways.

MTA promotes cytostatic effects rather than pro-apoptotic responses

Several publications have demonstrated both the cytostatic and pro-apoptotic effects of MTA in mammalian cells [10,26-28]. In our system, the proliferation curves and *in vivo* results suggested that MTA might be inhibiting cell proliferation and *in vivo* tumor growth through cytostatic rather than pro-apoptotic mechanisms. To further confirm this hypothesis we analyzed proliferation markers and the apoptosis levels of the tumor samples and performed *in vitro* experiments to investigate the effects of MTA in promoting apoptosis.

Immunostaining of the tumor tissues showed that samples from MTA treated mice had lower levels of cyclin D1 expression that closely correlated with a lower tumor proliferation index according to the Ki67 immunostaining (Fig 4A). Apoptosis within the tumor was measured by TUNEL assay and quantification of cleaved-caspase-3 positive tumor cells in paraffin sections. Our results showed that MTA-treated samples had slightly higher levels of apoptotic cells compared with control tumors (Fig 4B). In addition to this, it is known that angiogenesis is directly related to tumor growth. Interestingly, the intra-tumoral expression of VEGF, as well as the basal expression of VEGF in 37-31E cells was down regulated by MTA treatment (Fig 4C). Furthermore, these data correlated with a significant lower vessel density of MTA-treated tumor samples according to CD31 staining (Fig 4C).

We further analyzed the effect of MTA *in vitro*. 37-31E cells were treated with 10 μM MTA for different periods of time up to 48 h. At the molecular level the results showed that p-Bad and cleaved-caspase-3 levels did not change significantly over time, indicating the low levels of apoptosis (Fig 4D). However, MTA treatment promoted the de-phosphorylation of the mTOR downstream target ribosomal protein S6 and the down-regulation of the cyclin D1 protein levels, supporting the cytostatic effects of MTA (Fig 4D). Additionally, motility of 37-31E cells was also significantly reduced upon MTA treatment (additional file 1, panel B). Importantly, cell cycle analysis of melanoma cells harboring wild type RAS and BRAF (37-31E), $NRAS^{Q61L}$ mutation (SKMel147) and $BRAF^{V600E}$ mutation (UACC903) treated with 10 μM MTA for 48 h, showed an increased number of cells in G1 phase compared with untreated cells (6.6% ± 0.8% ($p < 0.05$), 8.9% ± 0.5% ($p < 0.05$) and 16.4% ± 0.7% ($p < 0.01$) respectively) (Fig 4E). Interestingly $BRAF^{V600E}$ mutant cells UACC903 showed the highest proportion of cells in G1 phase, indicating a slowed down cell cycle

Altogether, these results show evidences indicating that cytostatic effects on tumor cells mediate the reduction in melanoma tumor growth upon MTA treatment.

Discussion

Melanoma is the most serious form of skin cancer. If it is not recognized and treated early, the cancer can advance and spread to other parts of the body, where it becomes hard to treat and can be fatal. Recent studies

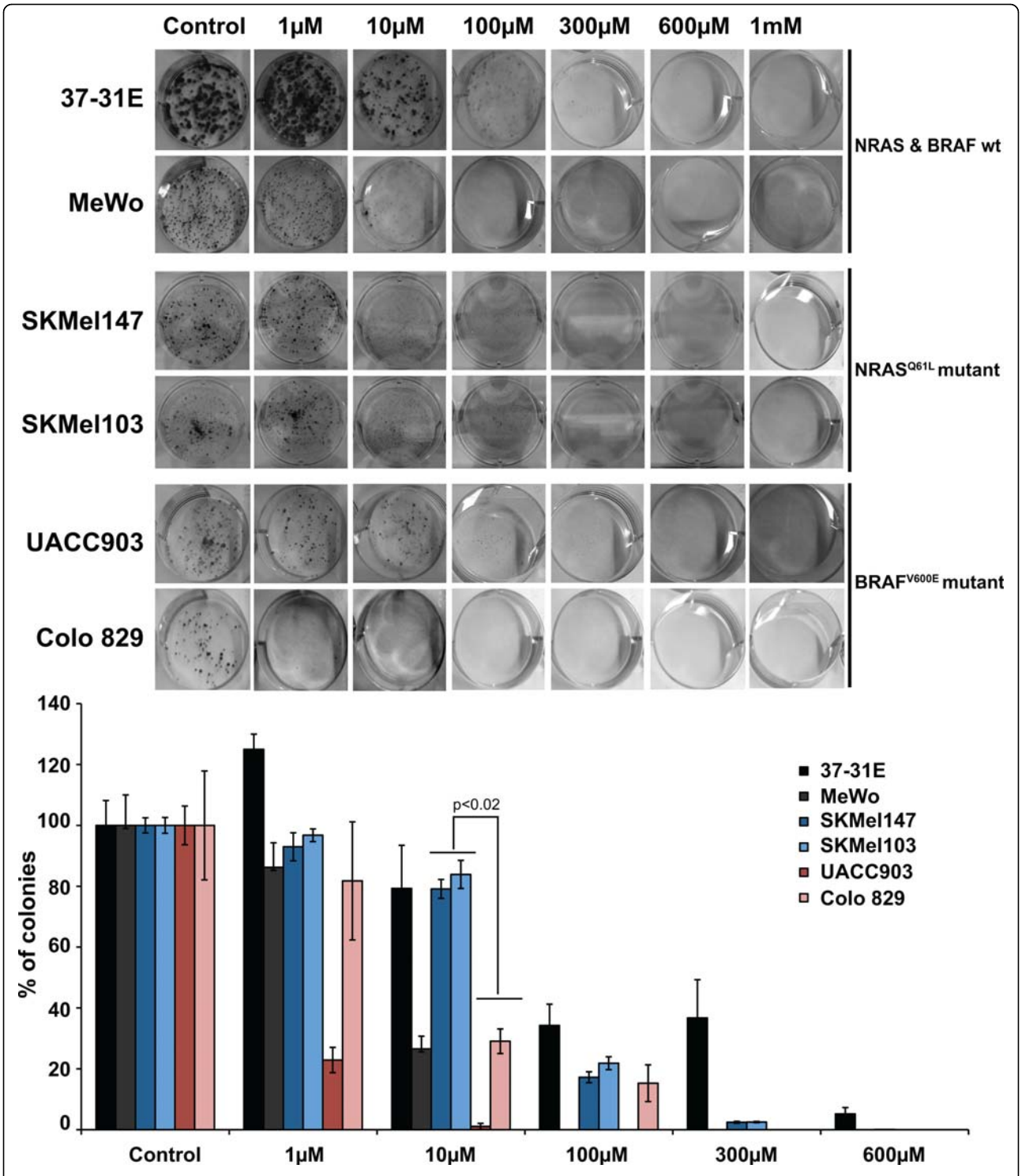


Figure 2 Methylthioadenosine (MTA) decreases melanoma cell viability. Clonogenic assays of 37-31E, MeWo, SKMel147, SKMel103, UACC903 and Colo 829 melanoma cells in complete medium with increasing concentrations of Methylthioadenosine (MTA). Three hundred cells were seeded clones were visualized by crystal violet staining and counted. Fresh media was changed every 48 h. Graphs represent the percentage reduction in the number of clones upon different treatments. *p-value* was calculated performing a Wilcoxon-signed rank test (Vassar Stats).

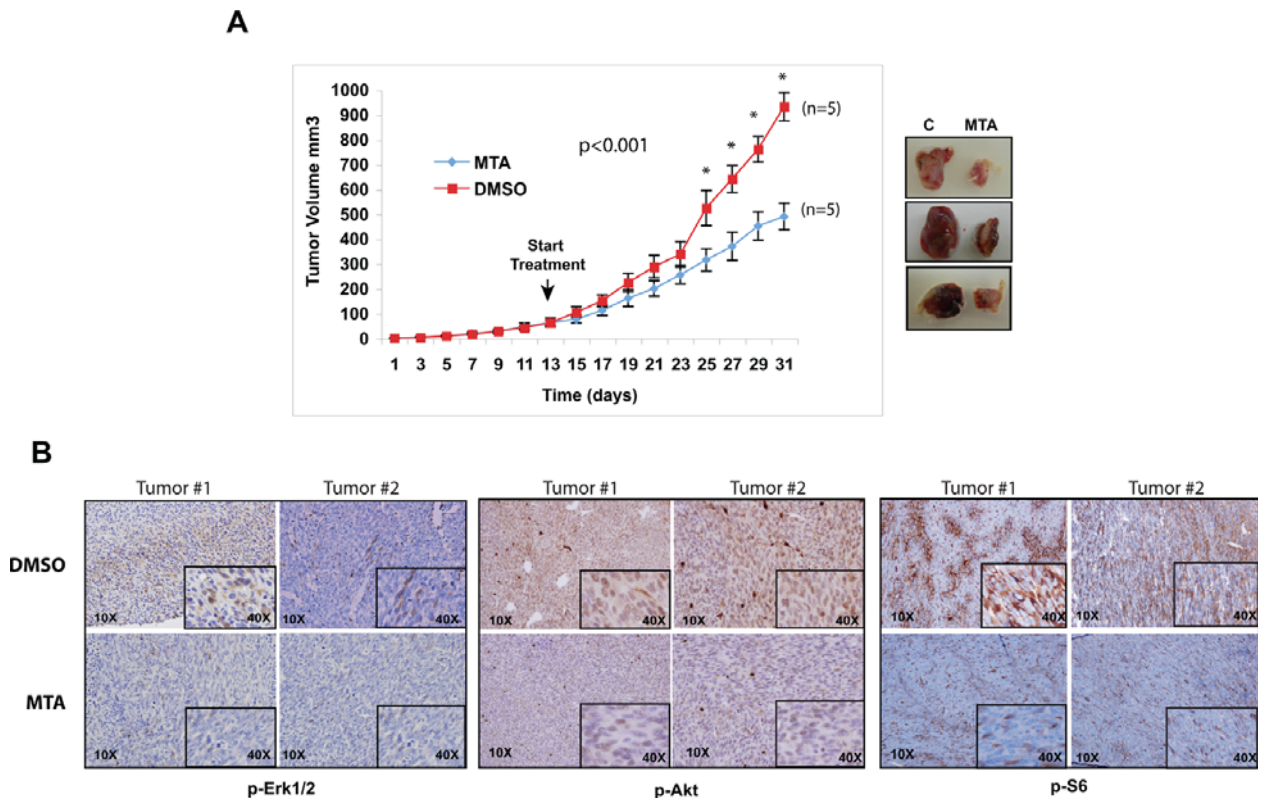
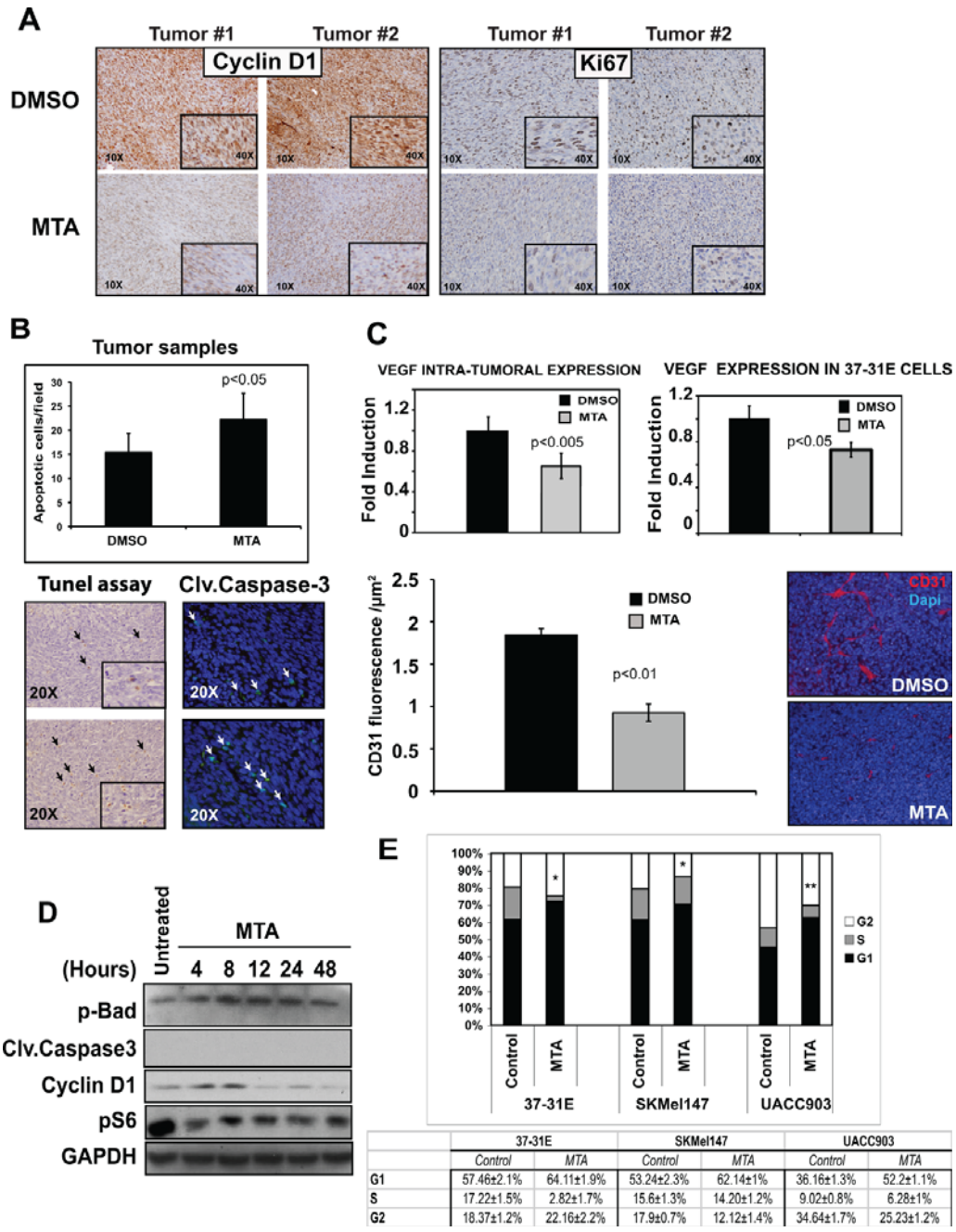


Figure 3 Methylthioadenosine (MTA) inhibits *in vivo* tumor growth and decreases the activation of RAS and PI3K tumor signaling pathways. **(A)** 37-31E melanoma cell line was subcutaneously injected into immunocompetent FVB/N mice. The animal groups were treated with DMSO (Control) (n = 7) or methylthioadenosine (MTA) (n = 7). Tumor size was calculated as described in materials and methods. *p*-values were calculated performing a *t*-student test. Representative photographs of three paired untreated and MTA-treated excised melanoma tumors are showed on the right. **(B)** Immunostaining of paraffin-embedded tumor sections showing the levels of p-Erk1/2, p-Akt and p-S6 (20×). Insets show a 40× detail of the picture. Representative images from two different untreated and treated tumors are shown.

have provided a much-improved understanding of melanoma biology, however, this knowledge has yet to be translated into effective treatment strategies. In this study, we investigated the therapeutic capabilities of MTA in melanoma treatment, a natural occurring nucleoside that has been shown to be effective in other tumor types [6,10,16,29,30]. Our results show that MTA inhibits *in vitro* cell proliferation, and viability in a dose dependent manner in a variety of human and mouse melanoma cell lines. Importantly, MTA treatment was also effective inhibiting *in vivo* tumor growth in a mouse melanoma xenograft model. Furthermore, the molecular analysis of the tumor samples and experiments performed with the cell lines indicated that in our model MTA has mostly cytostatic rather that pro-apoptotic effects.

Besides the inhibitory effects on the polyamine biosynthesis, MTA has been shown to exert other potent and specific pharmacological effects on cellular functions such as proliferation, apoptosis and modulation of the immune system [10,31,32]. Our current findings are in agreement with previous publications showing the inhibitory effects of MTA on proliferation and invasion (Fig 1 and additional file 1) of different types of tumor cells lines [33-35]. It is known that genetic mutations within tumor cells condition the drug response and this could be taken as an advantage in the design of more effective therapeutic approaches. Interestingly, BRAF mutant melanoma cell lines (UACC903 and Colo829) showed the highest sensitivity to MTA treatment, where concentrations of 10 μM of MTA reduced proliferation up to 70% (Fig 1D). Why BRAF mutant cells are more sensitive to MTA treatment is unknown and is an area of our current investigation. Nevertheless, the inhibition of cyclin D1 and proliferation by MTA might have a more pronounced effect in cells addicted to oncogenes with potent mitogenic effects. Additionally, the low levels of MTAP protein expressed in both cell lines might be contributing to the observed response.

In agreement with the *in vitro* results, MTA treatment reduced *in vivo* tumor growth by 45%. The molecular analysis of the tumors indicated that MTA treated



	37-31E		SKMel147		UACC903	
	Control	*MTA*	*Control*	*MTA*	*Control*	*MTA*
G1	57.46±2.1%	64.11±1.9%	53.24±2.3%	62.14±1%	36.16±1.3%	52.2±1.1%
S	17.22±1.5%	2.82±1.7%	15.6±1.3%	14.20±1.2%	9.02±0.8%	6.28±1%
G2	18.37±1.2%	22.16±2.2%	17.9±0.7%	12.12±1.4%	34.64±1.7%	25.23±1.2%

Figure 4 Methylthioadenosine (MTA) promotes cytostatic effects rather than pro-apoptotic effects in melanoma tumors and cells. **(A)** Tumor sections from mice treated either with DMSO or MTA were stained with cyclin D1 and Ki67 antibodies. Representative pictures are shown. **(B)** Quantification of apoptotic cells within the tumors. Paraffin-embedded tumor samples were subjected to TUNEL assay or stained against cleaved caspase-3. Graph shows the quantification of the TUNEL assay. Positive cells from ten fields (20×) per sample were quantified and the average number of cells per field was calculated. **(C)** Upper graph, quantification by qRT-PCR of VEGF levels in tumor samples. Lower graph, qRT-PCR of VEGF expression levels. 37-31E were untreated (Control) or treated for 48 h with 10 μM of MTA in complete medium. Microvessel's density quantification in xenografts. Graph shows CD31 fluorescence per μm^2. Representative pictures are showed on the right. *p*-values were calculated performing a *t*-student test. **(D)** 37-31E cells were treated with MTA (10 μM) for the time points indicated. Fifty micrograms of total lysates were resolved by PAGE-SDS. p-Bad, cleaved-caspase3, p-S6 and cyclin D1 protein levels are showed. GAPDH is used as a loading control. **(E)** MTA treatment induces a slowdown cell cycle G1 phase. Cells were grown in complete medium for 48 h in the presence or absence of MTA (10 μM). Cell cycle analysis was measured in triplicates using Cell Cycle Analysis Guava-Viacount reagent (Guava Technologies). Average of the three samples in each phase of the cell cycle are shown. *p*-values were calculated performing a *t*-student test (* = $p < 0.05$; ** = $p < 0.01$).

tumors had lower rates of proliferation according to the Ki67 and cyclin D1 levels, that correlated with lower levels in the PI3K and mTOR pathway activation and VEGF expression. While several studies have shown pro-apoptotic effects of MTA in tumor treatment [10,31], we observed a small increase of apoptosis in our tumor samples.

Recently, it has been described that MTA was able to induce the expression of growth factors and matrix metalloproteases in melanoma cells as well as enhance invasion and vasculogenic mimicry [5]. It is widely known that MTA inhibits methyltransferase enzymatic reactions and interferes with cellular pathways modulating cell signaling and protein expression [5,14,16,17]. In the mentioned study, the obtained data was generated at early time points of MTA treatment. Furthermore, the biological outcome in a long-term treatment, such as melanoma cell proliferation and the *in vivo* melanoma tumor growth, were not assessed. Our data show that the inhibitory effects of MTA on melanoma cell proliferation occur mostly after 48 h treatment. We believe that the biological response to MTA is dose dependent and cell type dependent. Indeed, several publications have shown that fibroblasts and normal hepatocytes have contrary responses to MTA compared to tumor cells. Moreover, in our system low concentrations of MTA (1 μM) promoted a slight increase in proliferation and viability, supporting a possible cell-type specific differential response to low concentrations of MTA. In mice, after intraperitoneal administration at 75 mg/kg, serum levels of MTA rapidly reached a peak of 28 μM rapidly and, at 30 minutes MTA was still at 10 μM [12]. In our hands a preliminary study of the bioavailability of MTA showed that plasma concentrations 20 min after i. p administration of this compound at 96 μmol/kg (equivalent to 30 mg/kg) were in the micromolar range (20-30 μM) (unpublished observations). In view of the efficacy of MTA reducing *in vivo* tumor growth, we speculate that the concentration reached within the tumor should be higher than 1 μM.

Previous studies have described a cytostatic effect of MTA on Mewo-LC1, Raji and R1.1 H cells [26-28]. According to our current observations in the xenograft model, MTA promoted cytostatic rather than pro-apoptotic effects. This result was confirmed by the *in vitro* experiments using the same cell line, where we found that MTA treatment induced the dephosphorylation of the downstream mTOR target S6 ribosomal protein, and the decrease of cyclin D1 protein levels. Importantly, we did not observe any molecular indication of apoptosis. Supporting these results, treatment of melanoma cells with MTA induced a cell cycle slow down in G1 phase. Interestingly, in agreement with the proliferation and viability results, UACC903 BRAF mutant melanoma cells showed the higher accumulation of cells in G1 phase.

MTA is a well-tolerated drug, devoid of the unwanted effects of other methyltransferase inhibitors. It has been administered previously in both acute and chronic experimental models of liver injury and systemic inflammation, showing efficacy and a safe profile [32] with an ID50 of 2.9-0.4 gm/kg (intramuscular) in rats [13]. In humans, MTA is also well tolerated [34,35]. Thus, MTA or any of its synthesized analogs would be good candidates for melanoma treatment in patients

Conclusions

Altogether, here we show the therapeutic potential of the naturally occurring nucleoside MTA in melanoma treatment. Importantly, our results demonstrate that MTA inhibits melanoma cell proliferation and *in vivo* tumor growth, supporting the antitumoral potential of MTA shown by others in other type of tumors. The data also suggest that $BRAF^{V600E}$ mutant cells appeared to be more sensitive to the cytostatic effects mediated by MTA. This study outlines an efficacious and well-tolerated therapeutic candidate for melanoma intervention.

Additional material

Additional file 1: Figure S1. A) MTA does not inhibit proliferation and viability of normal cells (MEFs). Cell proliferation of mouse embryo fibroblasts (MEFs) in response to increasing concentration of MTA. Medium was changed adding fresh MTA every 48 h. Proliferation assays were performed by counting the number of viable cells using Guava-Viacount reagent (Guava Technologies) in a cell counter (Viacount) at the time points indicated. Clonogenic assays using MEFs in complete medium with increasing concentrations of Methylthioadenosine (MTA). Three hundred cells were seeded. Clones were visualized by crystal violet staining and counted. Fresh media was changed every 48 h. Graphs represent the percentage reduction in the number of clones upon different treatments. p-value was calculated performing a Wilcoxon-signed rank test (Vassar Stats). **(B) MTA inhibits motility of 37-31E melanoma cells:** 37-31 melanoma cells were seeded in a 24 multi-well plate trans-wells (Corning). Following treatment (either DMSO or MTA 100 μM for the times indicated) cells were washed with PBS and fixed with 1% glutaraldehyde in PBS. Cells were then stained with an aqueous solution of 0.1% crystal violet. After destaining in water, non-migrating cells in the top of the trans-well were removed, and stained migrating cells in the bottom of the trans-well were destaining with PBS containing 0.2% Triton X-100. The O.D. was then measured at 590 nm (Gillies et al., 1986). All the experiments were done in triplicates. p-values were calculated performing a t-student test (*) = $p < 0.01$.

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Authors' contributions
PAP did most of the experimental work and data analysis. AP was involved in the animal work. JG She was involved in the experimental work. JHL, RG and TM performed the pathological and immunohistochemical analysis. JC was involved in the analysis and discussion of the data. MAA contributed with some reagents, analyzed and discussed the data. JAR conceived and designed the study, coordinated the work, anlayzed the data and wrote the paper. All the authors approved and read the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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