Ubiquitin-dependent control of Kinase Suppressor of Ras 1 (KSR1) signaling by the RING ligase praja2.

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LIST OF ABBREVIATIONS

KSR1 Kinase Suppressor of Ras 1
MAPK Mitogen-Activated Protein Kinases
MAP3K MAPK kinase kinase
MAP2K MAPK kinase
EGF epidermal growth factor
EGFR EGF receptor
GTP guanosine triphosphate
PKC Protein Kinase C
GPCR G-protein couple receptor
PKA Protein Kinase A
NGF Nerve Growth Factor
cAMP cyclic-AMP
GST Glutathione S-transferase
Kinase Suppressor of Ras 1 (KSR1) is an evolutionally conserved protein kinase that plays a fundamental role in mitogenic pathway. In response to Ras activation, KSR1 assembles a tripartite kinase complex that optimally transfers signals generated at cell membrane to downstream ERK signaling. The role of KSR1 in Ras signaling has been largely explored. However, the impact of attenuating signals on KSR1 was still elusive. Here, I contributed to identify a novel mechanism of ERK attenuation based on the ubiquitin-dependent control of KSR1. Stimulation of membrane receptors by growth factor induced a rapid poly-ubiquitination of KSR1, which paralleled the decay of ERK signaling. We identified praja2 as the principal E3 ligase that ubiquitinates KSR1. Interfering with praja2 expression or activity impeded KSR1 ubiquitination and sustained ERK signaling.

Thus, the dynamic interplay between the ubiquitin system and scaffold components of the Ras pathway contributes to shape the profile of ERK signaling, profoundly impacting on fundamental aspects of cell behavior.
INTRODUCTION
1.1. Mitogen-Activated Protein Kinases (MAPK) pathway and their scaffold proteins

Several responses to hormones, or any extracellular stimulus, are transmitted to various intracellular targets through a series of protein networks that regulate and govern many cellular processes. The extracellular ligands interact with receptors on the cell membrane, which in turn activate the intracellular second messengers, thus initiating a cascade of signal transduction towards several target proteins. Among the repertoire of the cascades of signaling molecules there is a family of protein kinase known as mitogen-activated protein (MAP) kinase. All these cascades must contain at least three protein kinases in series that culminate in the activation of a multifunctional MAP kinase (Lewis TS. 1998). MAP kinases are the main components of the pathways that control embryogenesis, differentiation, proliferation, and cell death. The mammalian MAP kinases consist of a cytoplasmic protein serine\treonine kinases that include the extracellular signal-regulated kinase (ERK) family, the p 38 family, the c-Jun N-terminal kinase family. Each MAP kinase is regulated by a phosphorylation cascade that starts with a MAPK kinase kinase (MAP3K) that phosphorylates and activates a MAPK kinase (MAP2K). MAPK kinase (MAP2K) phosphorylates and activates causing the phosphorylation of several downstream substrates such as transcription factors and other functional proteins. In ERK1\2 pathway these kinases are called Raf, MEK, and ERK (Figure 1);
Figure 1. MAPK specificity  Diverse extracellular inputs active the Ras/Raf/MEK/ERK cascade and each can give a different cellular outcome

The amplitude and the attenuation of the signal stimulated by MAP kinases are tightly regulated. In particular, the cascade of phosphorylation of the ERK1/2 pathway is amplified at the step of RAF phosphorylation on MEK, because MEK is about 100 times more abundant than the other two kinases (RAF and ERK) (Huang C-YF. 1996; Ferrell Jr JE. 1996).

This pathway is activated by protein tyrosine kinase receptors, like EGF receptor (EGFR) or VEGF receptor (VEGFR) (Pearson G.; 2001). In response to the ligand stimulus, the receptors dimerize, inducing trans-phosphorylation by intrinsic tyrosine kinases. This event leads to the recruitment of some proteins and to the activation of Ras. Ras is a GTPase, which hydrolyses guanosine triphosphate (GTP) to guanosine diphosphate (GDP). Active GTP-Ras, kicks off the signal. There are three Ras isoforms in mammals, namely H-Ras, K-Ras and N-Ras and it has been shown that mutations in the Ras gene are found in about 30% of human cancers, such as melanoma and biliary cancer (Vakiani E. et al. 2011).

Ras activates Raf kinase, recruiting it to the membrane. There are three known isoforms of Raf, called A-Raf, B-Raf and C-Raf (also known as Raf-1), each of them has distinct functions
(Wellbrock C. 2004). Each isoform contains three conserved regions, termed CR1, CR2, and CR3, where it is located the kinase domain. In turn, Raf becomes active and catalyses phosphorylation and activation of MEK1 and MEK2, which in turn activate ERK1/2. These are ubiquitous protein kinases of a 44 and 42 kDa, respectively. ERK1 and ERK2 have 85% of identity, especially in the region involved in binding substrates (Boulton TG.; 1991). Nevertheless, some studies have shown that functions of the two isoforms are different. Indeed, mice ERK2-/- are not viable (Yao Y. et al.; 2003), while mice ERK1–/- seem to have only a defect in the maturation of CD4+ and CD8+ cells (Pagès G. et al; 1999). Once activated, ERKs dimerize and translocate to the nucleus, where they phosphorylate transcription factors, or remain in the cytosol where they phosphorylate substrates in multiple cellular compartments (Matthew D. Brown; 2009) (Figure 2).

Since Raf can translocate to the plasma membrane Raf/MEK/ERK pathway may be also activated by some stimuli such as Src and PKC kinases, and by GPCR activation. It is shown that isoproterenol (a drug that acting through a GPCR, increases the intracellular level of cyclic AMP) treatment is able to increase the level of the active form of ERK in human embryonic kidney cells (Daaka Y.; 1997 Copik AJ1; 2015).

Instead, the effects caused by cAMP-dependent protein kinase A (PKA) are disputable, because some evidence indicate that PKA reduces Raf-1 activity (Wu J et al. 1993; Hafner S. et al. 1994; Kikuchi A. et al. 1996; Mischak H. et al. 1996). On the other hand, studies in neuronal cells demonstrated that PKA phosphorylates Rap1a, which increases ERK phosphorylation, through activation of B-Raf (Vossler MR. et al. 1997 - Grewal SS. et al. 2000).

Temporal modulation of ERK cascade arouses distinct cellular responses (Murphy LO et al. 2006). In pheocromocytoma cells (PC12), different responses are observed after different stimuli that activate the same MEK/ERK pathway. PC12 cells proliferate in response to EGF, while nerve growth factor (NGF) induces differentiation (York RD1 1998). Further investigation has shown that EGF-dependent MAPK signaling is transient, lasting minutes from activation, while NGF-dependent signaling is sustained for hours from stimulation (Marshall CJ1 1995).

So, who regulates the signal duration and intensity? And also, which upstream stimulus leads to the activation of every specific downstream signal?

The mechanisms that regulate these phenomena have been unknown for long time. Presently, there are some evidences that
the scaffold proteins may be the answers to these questions. The presence of scaffold proteins as relevant components of MAPK pathway was studied firstly in the yeast S.Cevisiae, where mutations in Ste5, a protein involved in the three-MAPK cascade assembly, destroyed the response to pheromone (Choi et al. 1994; Marcus et al. 1994). There are evidences that also in mammalian MEK\ERK MAPK pathway the scaffold proteins have an important role for the correct activation, the signal propagation and turn off of the cascade. Moreover, scaffold proteins regulate and compartmentalize the signal diffusion to a specific subcellular location (Roskoski, 2012). Scaffolds are thought to minimize crosstalk with other signaling cascades (Dhanasekaran et al. 2007) and similarly mediate crosstalk of different pathways (Kolch, 2005) and protect kinases from phosphatases action (Perlson et al. 2006).

In the last years, different mammalian scaffold proteins have been isolated and characterized: KSR1 and KSR2, β-arrestin, paxilin MP1, MORG1, Sef and IQGAP1, but are located in different cellular districts, and so, they direct the signal amplification on different cellular compartments. In this context, KSR1/2 binds the kinases in the cytosol, β-arrestin in clathrin vesicles, MP1 active complex in late endosomes and Sef locates the kinase complex in the Golgi apparatus. Thus, different stimuli can activate a complex formation with a different scaffold protein. When the stimulus starts from an activated GPCR and is directed to early endosomes, β-arrestin captures the kinases and compartmentalizes there the signal (Figure 2).
Figure 2. Spatial regulation of MEK/ERK signaling by protein scaffolds. Scaffolds can regulate the subcellular distribution MAPK proteins. Both IQGAP1 and KSR bind to Raf, MEK and ERK and localize the kinases to the plasma membrane, while MP-1 localizes the kinase complex to endosomes. After activation, ERK phosphorylates the downstream targets, or translocate to the nucleus and activate nuclear targets. KSR binds ERK dimers and restricts them to cytosolic targets. Like MP-1, β-arrestins localize Raf, MEK and ERK to endosomes. However, once activated, ERK cannot translocate to the nucleus and phosphorylates mostly cytosolic targets by an unknown mechanism. Sef restricts MEK and ERK to the Golgi and, like β-arrestin, prevents translocation of ERK to the nucleus, limiting it to cytosolic substrates. GPCR, G-protein coupled receptor; RTK, receptor tyrosine kinase.
1.2 Kinase Suppressor of Ras1 (KSR1)

The KSR family proteins consist of two members namely KSR1 and KSR2. They are widely conserved from invertebrate to mammals and the similarity of amino acids between them is approximately 60%. The structure of these proteins consists of 5 domains called CA1-CA5. The CA1 domain is located in the N-terminus and it is involved in the formation of a KSR1-MEK-RAF complex; it comprises 40 amino acids which are exclusive to KSR1, but absent from KSR2; CA2 is a proline-rich domain with unknown function; CA3 is a cysteine rich domain, needful for membrane recruitment by phospholipids (Michaud N.R. 1997); CA4 is a serine/threonine-rich region containing an FXFP (Phe-Xaa-Phe-Pro) motif that is an ERK docking-site (Kolch W. 2005) and CA5 is highly homologous to the kinase domain of RAF proteins (see Figure 3) and, for this reason, it is considered a putative kinase domain which lacks the conserved lysine residue, required for phosphorylation (Kolch W. 2005) (Figure 3). Furthermore a new domain of KSR1 composed by a coiled-coil (CC) and a sterile α(SAM) motifs, thus called CC-SAM, has been recently characterized. This domain targets KSR-1 in response to growth factor at the plasma membrane, and binds directly to various micelles and bicelles in vitro. (Koveal D. 2012).

Figure 3. KSR structure. KSR is composed by 5 domains called CA1-CA5. The CA1 domain is responsible of the formation of a KSR1-MEK-RAF complex; CA2 is a proline-rich domain with unknown function; CA3 is a cysteine rich domain, needful for membrane recruitment with phospholipids; CA4 is a serine/threonine-rich region containing an FXFP (Phe-Xaa-Phe-Pro) motif that is an ERK docking-site and CA5 is a putative kinase domain in which the conserved lysine residue.
KSR1 and KRS2 were initially characterized as scaffold proteins involved in different pathways. KSR2 has several functions; it contains a region of 63 amino acids between the portion C3-C4, which is a specific region for binding AMPK and calcineurin. Calcineurin, when activated by intracellular calcium, binds KSR2 and activates ERK signaling (Costanzo-Garvey DL. 2009; Dougherty MK. 2009). KSR2 gains an important function in energy metabolism regulation, as KSR2 knockout mice show an obese phenotype (Brommage R. 2008).

On the other hand, KSR1 is recruited in the Ras/Raf/MAPK signaling cascade. In un-stimulated cells, C-TAK1 (Cdc25C-associated kinase 1) phosphorylates KSR1 at Ser392. Hence, KSR1 is sequestered in the cytosol, where it constitutively interacts with MEK and ERK. Upon growth factor stimulation, Ras is activated and triggers dephosphorylation of KSR1 at Ser392 by PP2A (protein phosphatase 2A). After that, KSR1 shifts to the cell membrane, where it forms a complex with Raf, MEK and ERK. KSR1 thus potentially makes easy the phosphorylation of Raf, MEK and ERK, facilitating the MAPK signaling, as well as the phosphorylation of multiple substrates in the cytoplasm and nucleus. Through this way, KSR1 regulates proliferation, cell cycle and apoptosis (Muller J. 2001; Roy F. 2002; Ory S. 2003) (Figure 4).
Figure 4. KSR1 regulation in the Ras/Raf/MAPK pathway. In resting conditions, KSR1 is sequestered in the cytosol through 14-3-3 protein binding, after phosphorylation by C-TAK1 at Ser297 and Ser392. Meantime, KSR1 constributes with MEK and ERK. Under stimulated conditions, it activates Ras that triggers dephosphorylation of KSR1 at Ser392 by PP2A, leading to the release of 14-3-3 protein from its binding sites. KSR1 switches to the cell membrane, where it forms a complex with Raf, MEK and ERK, and promotes phosphorylation of downstream substrates.

However, different groups found conflicting results on the role of KSR in the regulation of MAPK. Denouel-Galy showed that the MEK subpopulation complexed with exogenous KSR1in COS-1 cells or PC12 cells have no kinase activity because KSR1 blocks MEK in an inactive form. This block has a surprising biological effect: KSR1 did not transform NIH3T3 cells, and, furthermore, reduced Ras-induced transformation. Similarly, KSR1 inhibited the proliferation of embryonic neuroretinal cells induced by Ras (Denouel-Galy, A. 1998).

Protein scaffolds must be expressed in the cell at certain levels. Indeed, KSR1 may have different effects in the regulation of MAPK signaling according to its intracellular concentration. In fact, KSR1 at low concentrations can increase the Ras downstream signaling, while high levels of KSR1 have inhibitory effects on Ras pathway (Cacace AM. ; 1999).

KSR1 is also an important regulator of the immune response. Thus, KSR1 plays a role in the protection from the inflammation,
bacterial infections, and in T cells proliferation. KSR1−/− are more susceptible to the manifestation of colitis induced by TNF alpha (Yan, F. 2004; Yan, F. 2001) and to infection by Pseudomonas aeruginosa (Zhang, Y. 2011). Perhaps, this was a consequence of KSR1-mediated cytotoxic response of natural killer cells. Furthermore, in these mice the attenuation of the ERK pathway also causes a reduction of T-cell proliferation (Nguyen, A. 2002). However, KSR1 has not only the role of scaffold protein. KSR1 has been classified as inactive pseudo-kinase. However, in vitro studies have shown that KSR1 owns a real kinase activity and it is capable both of auto-phosphorylation and of MEK phosphorylation (Goettel, J.A. 2011). Furthermore, KSR1 phosphorylates other substrates, even if not bound to MEK (Xing, H.R. 2004). In addition, KSR1 is able to regulate the temporal duration of the signal. McKay and collaborators demonstrated that KSR1 is subjected to phosphorylation and inhibition by activated ERK. In this circumstances, the scaffold is temporarily inactivated and the multikinase complex is disassembled (McKay, M. et al. 2009) (Figure 5).

Figure 5. KSR1 regulates the intensity and duration of ERK cascade. In resting cells, KSR1 binds MEK and keeps it away from Raf (A). In stimulated cells, KSR1 facilitates the Raf/MEK interaction and first increases the signal transmission (B) and then attenuates the signal. Active ERK, in fact, can phosphorylate KSR1 facilitating the disruption of the KSR1 scaffold complex (C and D).
1.3 The ubiquitin proteasome system

The amount of protein within the cell is usually kept constant or varied according to the needs of the latter. Dysregulation in the amount of specific proteins can lead to the development of diseases or disorders, including cancer. The balance between the synthesis and degradation of proteins is regulated by the ubiquitin-proteasome system. Ubiquitination is crucial for several physiological processes, including cell survival, differentiation, innate and adaptive immunity.

The cellular functions of ubiquitination include proteolytic and non-proteolytic roles such as proteasomal degradation of proteins, receptor internalization and down regulation, intracellular trafficking, inflammatory signaling and assembly or disassembly of multi-protein complexes (Grabbe C. et all 2011). Thus, a wrong regulation of ubiquitination might have pathological consequences, as degenerative disorders and cancer.

Ubiquitination is largely mediated by ubiquitin ligases, which mark their substrates for, with a series of ubiquitin molecules (Hershko et al. 1998). The conjugation of ubiquitin molecules to substrates requires coordinated action of the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme E2 and the E3 ligase. The E3 ligase enzyme associates with the substrate and thereby determines the specificity in degradation or inactivation of various substrates (Ciechanover, 1998) (Figure 6). As already mentioned, ubiquitination does not always lead to protein degradation. Indeed in recent years have been identified more than 100 de-ubiquitination enzymes (DUB) that remove ubiquitin from substrates, making them available again (Amerik A.Y. et al. 2004; Huang OW et al. 2013)(Figure 6).

The RING finger ubiquitin E3 ligases contain a characteristic cysteine-rich zinc binding domain defined by a pattern of conserved cysteine and histidine residues. These ligases efficiently catalyze poly-ubiquitination of given substrates (Joazeiro et al. 2000). Characteristic of RING finger ubiquitin E3 ligases is the ability to mediate degradation of their substrates, by promoting their own ubiquitin-dependent degradation (Lorick et al. 1999).
Figure 6. Schematic representation of ubiquitin system. The conjugation of ubiquitin molecules to substrates requires coordinated action of three enzymes: the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme E2 and the E3 ligase, that associates the ubiquitin molecules to the substrates. Once ubiquitinated, the proteins can be degraded by the proteasome or de-ubiquitinated by a specific DUBs enzyme.

1.4 RING ligase Praja2

Praja2 belongs to the Ring H2 finger E3 ubiquitin ligase subfamily. It contains, in fact, at COOH-terminal end (aa 629-678) a characteristic sequence domain known as the RING-H2-finger motif (Freemont, 1993). The RING-H2-finger motif is similar to the RING-finger motif, and Cys4 in the RING-finger motif is replaced by His (Freemont, 1993).

Praja E3 ligase family is composed by two members, namely praja1 and praja2, that share high sequence homology. praja2 gene is localized on the long arm of human chromosome 5 (5q21) and its transcript encodes for a protein of 708 amino-acidic with an estimate weight of 78 KDa (Nakayama et al. 1995). But SDS-polyacrylamide electrophoresis gel show that the molecular mass of praja2 is estimated to be about 140 kDa. This discrepancy between the expected and observed molecular masses might be explained by anomaly of protein migration rates on SDS-polyacrylamide gel (Ohara and Teraoka, 1987). Instead, praja1 gene
mapped on human chromosome X. Northern blot analysis identify a 2.7 Kbp ribonucleotide transcript, coding for a protein of 643 amino-acids, with an estimate weight of 71 kDa (Ping et al. 2002). Praja1 and praja2 show high sequence homology, more or less of 52.3% of identity and their COOH terminal domains contain a RING H2 Finger domain highly conserved. It is demonstrated that in mice, praja1 has an important role in neuronal plasticity that is the basis for learning and memory (Ping et al. 2002), in fact praja1 expression shows learning-specific induction in the basolateral complex of the amygdala during formation of fear memory (Stork et al. 2001). Structural data and sequence analysis identified in praja2 COOH terminal domain (aa 583-600) a particular motif, highly conserved among all the praja family members, encoding an α-amphipathic helix. This domain is characteristic of AKAPs family proteins, and is known to be fundamental for PKA anchoring (Figure 7).

Figure 7. Schematic representation of praja proteins. Praja1 and praja2 are composed respectively by 643 and 708 aminoacids. Both proteins are characterized by a RING-H2 motif (in rose) and a putative amphipatic helical wheel (in yellow) at COOH-terminal.

Following axotomy, axonal degeneration and neuronal cell death correlate with strong down-regulation of praja2 expression levels in rat (Nakayama et al. 1995). Thus, regulating synaptic communication and plasticity, praja family proteins play a crucial role in neuronal activity and development (Nakayama et al. 1995). praja2 plays an important role in several molecular mechanisms that mostly involve the cAMP-dependent signal transduction pathway. First of all, praja2 controls the stability of mammalian regulatory subunits of Protein Kinase A settling the strength and duration of PKA signal output in response to cAMP, so it is required for efficient nuclear cAMP signaling and PKA-
mediated long-term memory (Lignitto et al. 2011). Moreover, praja2 is involved in cAMP-induced neurite outgrowth, promoting proteasomal degradation of NOGO-A, a major inhibitor of neurite outgrowth in mammalian brain (Sepe et al. 2014) (Figure 8).

![Model of praja2–NOGO-A pathway](image)

**Figure 8. Model of praja2–NOGO-A pathway.** Under basal conditions, NOGO-A inhibits neurite outgrowth. Activation of adenylate cyclase (AC) by BDNF or by Forskolin (Fsk) increases cAMP levels and dissociates PKA-holoenzyme. Active PKA catalytic subunit phosphorylates praja2, which in turn ubiquitinates and degrades NOGO-A through the UPS. The drop in NOGO-A levels promotes neurite outgrowth (Sepe et al. 2014).

In addition, praja2 has a role in a tumor growth, indeed it ubiquitinates and degrades MOB1, a core component of NDR/LATS kinase and a positive regulator of the tumor-suppressor Hippo cascade. This degradation attenuates the Hippo cascade, controls the organ size and sustains glioblastoma growth in vivo (Lignitto et al. 2013) (Figure 9).
Figure 9. Schematic representation of the role of praja2 in the Hippo pathway. Praja2 ubiquitinates MOB1 and degrades it thorough the proteasome system. MOB1 degradation attenuates LATS1/2 kinase activity, preventing phosphorylation and inactivation of YAP. Un-phosphorylated YAP accumulates in the nucleus and drives gene transcription, promoting cell proliferation and tumor growth.

On the other hand, praja2 is markedly over-expressed in differentiated thyroid cancer, and its levels inversely correlate with the malignant phenotype of the tumor (Cantara et al. 2012). Finally, praja2 also seems to be involved in the regulation of insulin secretion. Indeed, in response to glucose stimulation, praja2 ubiquitinates and degrades p35, activating calcium signaling and promoting insulin secretion (Sakamaki J. et al. 2014).

Many RING proteins have functions unknown or unrelated to the ubiquitination in evident manner. Interestingly, several RING finger genes have been found mutated in human diseases (Mattson et al. 2001; Kitada et al. 1998). For instance, the parkin gene was found mutated in autosomal recessive familial juvenile Parkinsonism (Kitada et al. 1998).
AIM OF THE STUDY
Kinase Suppressor of Ras 1 (KSR1) is an evolutionally conserved protein kinase that plays a fundamental role for correct activation, propagation and attenuation of the mitogenic pathway (Roskoski et al. 2012). In response to Ras activation, KSR1 assembles a kinase complex that optimally transfers signals generated at cell membrane to downstream ERK signaling.

Ubiquitination is a post-translational mechanism that controls the activity/stability of a wide array of cellular target proteins. By modulating protein stability, the ubiquitin pathway controls key physiological processes, such as survival, differentiation and cell proliferation (Grabbe C. et all 2011).

Our laboratory is involved in the regulation of the ubiquitin pathway by the E3 ubiquitin-ligase praja2. Recently, we identified praja2 as a component of the complex assembled by KSR1. During the PhD program, my principal aim was to understand if KSR1 and praja2 formed a complex in living cells and how the complex regulates MAPK signaling. More specifically, I focused my attention on the following issues:

- Verify the existence of KSR1-praja2 complex in living cells.
- Test if praja2 ubiquitinates KSR1 in vitro and in vivo.
- Define the functional relevance of praja2 in KSR1-dependent mitogenic pathway.
RESULTS
2.1 praja2 and KSR1 form a stable complex

To gain insights into praja2 action, I tested if praja2 interacts with protein/adaptors involved in mitogenic signaling. To this end, I performed co-immunoprecipitation assays using antibodies directed against distinct components of the ERK pathway. This analysis allowed the identification of Kinase Suppressor of Ras 1 (KSR1) as a relevant interactor of praja2. A proteomic screening previously identified praja2 as component of a macromolecular complex that includes KSR1 (Dougherty M.K. et al. 2009).

First, I investigated whether endogenous praja2 and KSR1 interact in cells. Lysates from HEK293 cells were immunoprecipitated with anti-praja2 or with non-immune IgG. Precipitates were immunoblotted with anti-KSR1 and anti-praja2 polyclonal antibody. As shown, an endogenous praja2-KSR1 complex could be detected (Figure 10).

Figure 10. praja2 binds KSR1. Lysates (2mg) from HEK293 cells were immunoprecipitated with anti-praja2 or non-immune IgG. Precipitates were immunoblotted with anti-KSR1 or anti-praja2 polyclonal antibody.
2.2 praja2 directly interacts with KSR1

As shown before, praja2 interacts with KSR1 protein. To demonstrate whether this binding is direct, I performed a pull down assay using recombinant proteins. Thus, a fusion protein carrying the full-length praja2, appended to the C-terminus of glutathione S-transferase polypeptide (GST), pulled down in vitro translated $^{35}$S-labeled KSR1 (Figure 11).

![Figure 11. praja2 directly interacts with KSR1 in vitro.](image)

In vitro translated, $^{35}$S-labeled KSR1 was subjected to pull-down assays with purified GST or GST-praja2 fusions.
2.3 praja2-KSR1 binding is regulated by serum

Next, I assessed whether praja2-KSR1 interaction is regulated by serum. Lysates from growing or serum-deprived HEK293 cells were immunoprecipitated with anti-praja2 or non-immune IgG. Precipitates were immunoblotted with anti-KSR1 or anti-praja2 polyclonal antibody. As shown in Figure 12, the praja2-KSR1 interaction is regulated by serum. Indeed, praja2-KSR1 complex was more evident when cells have been grown in the presence of serum.

Figure 12. praja2-KSR1 binding is regulated by serum. Lysates (2mg) from growing or serum-deprived (18h) HEK293 cells were immunoprecipitated with anti-praja2 or non-immune IgG. Precipitates were immunoblotted with anti-KSR1 or anti-praja2 polyclonal antibody.
2.4 Endogenous KSR1 and praja2 co-localize in Human Embryonic Kidney 293 cells.

Next, to demonstrate that KSR1 and praja2 are located within the same cell compartment, I analyzed the localization of both proteins in Human Embryonic Kidney 293 cells (HEK293). To this end, I performed double immunofluorescence using anti-KSR1 and anti-praja2 antibodies. As suspected, the results indicate that both proteins have a partial co-localization within the cytoplasm and at the perinuclear region, as confirmed by the Pearson’s statistic analysis (Figure 13).

![Image](image-url)

Figure 13. praja2 and KSR1 co-localize in Human Embryonic Kidney (HEK) 293 cells. HEK293 cells were subjected to double immunostaining with anti-ksr1 and anti-praja2 antibodies. Images were collected and analyzed by confocal microscopy. Magnification of selected areas is shown (insets). Co-localization Pearson’s coefficient praja2-KSR1=0.54. Scale bar: 10 μm.
2.5 Mapping the praja2 binding domain on KSR1.

The data above demonstrate that KSR1 and praja2 form a stable complex in lysates and partly co-localize within the same sub-cellular compartment. To determine whether praja2 ligase activity is required for KSR1 binding, HEK293 cells were transiently transfected with flag-tagged praja2 or with a flag-tagged praja2RING mutant. The mutant protein carries cysteine to alanine substitutions within the RING domain (cys634 and cys671) (praja2rm) that interferes with the transfer of the ubiquitin moiety from E2 to the substrate. Lysates were immunoprecipitated with anti-flag antibody. Results show that praja2 ligase activity was not necessary for KSR1 binding, since praja2rm binds KSR1 as wild type protein. To identify the domain of praja2 that mediates the binding to KSR1, I generated a series of deletion mutants lacking distinct segments of praja2. Flag-tagged praja2 mutants were co-expressed with KSR1 in HEK293 cells. Lysates were immunoprecipitated with anti-KSR1 antibody, and immunoblotted with anti-flag antibody.

The results show that the mutant lacking aminoacids from 402 to 708 was not able to bind KSR1, suggesting that residues 402 to 531 of praja2 are most likely required for optimal binding to KSR1 (Figure 14).
Figure 14. praja2 402-531 residues mediate the binding to KSR1. Schematic representation of human praja2 and its deletion mutants. RING domain and KSR1 binding domain are shown in grey and in blue (upper panel), respectively. HEK293 were transfected with flag-praja2, praja2 RING mutant (C634,671A; praja2rm), or with each of the three deletion mutants (Δ631-708, Δ531-708, Δ402-708). Cells were treated with MG132 (10 μM) for 6 hours before harvesting. Lysates were immunoprecipitated (IP) with anti-KSR1 and immunoblotted with anti-Flag and anti-KSR1 antibodies.
2.6 Mapping the KSR1 binding domain on praja2

Next, I analyzed which part of KSR1 interacts with praja2. To this end, overlapping 25-mer peptides derived from human KSR1 were spotted onto a membrane and overlaid with purified GST-praja2\textsuperscript{431-531} fusion protein, as previously described (Stefan E. et al. 2011). I identified one binding motif at the C-terminus of KSR1 (DLQEROSFL) as the core region required for praja2 binding (Figure15A). As expected, a recombinant protein carrying the core peptide of KSR1 fused at the C-terminus of the GST polypeptide was sufficient in binding flag-praja2 \textit{in vitro} (Figure15B). The peptide is located at the surface of the predicted KSR1 structure, supporting the findings of a direct interaction with praja2 (Figure15C).
Figure 15. KSR1 necessary binding site for praja2 is on the surface of protein.

(A) Spotted peptides (25 mer, 15 amino-acids overlap) of KSR1 were overlaid with recombinant GST-praja2\textsubscript{1-531} followed by immunoblotting with anti-GST. The potential binding amino acids are shown in red (upper panel).

(B) HEK293 were transfected with flag-praja2 and the lysates were incubated over night with GST fusion protein containing a peptide sequence (GST-pep) and a scramble short sequence (GST-cnt). The proteins were immunoblotted with anti-Flag and anti-GST antibodies. (C) KSR1 predicted structure (down panel). The potential praja2 binding site is shown in red.
2.7 praja2 ubiquitinates KSR1

E3 ubiquitin ligase praja2 binds KSR1. Therefore, I tested if praja2 acting as E3 ligase ubiquitinates KSR1. HEK293 cells were co-transfected with praja2-flag, praja2rm-flag, and HA-ubiquitin vectors. Lysates were immunoprecipitated with anti-KSR1 antibody. The precipitates were immunoblotted with anti-HA, anti-KSR1 and anti-flag antibodies. The data indicates that expression of praja2, but not of praja2rm, induced accumulation of poly-ubiquitinated KSR1 molecules (Figure 16A). Next, I analysed if the activation of tyrosine kinase receptor (RTK) modulates KSR1 ubiquitination. Stimulation by epidermal growth factor (EGF) induced a strong KSR1 polyubiquitination (Figure 16B). Expression of praja2rm dramatically impaired ubiquitination of KSR1 induced by EGF (Figure 16C). An in vitro ubiquitination assay demonstrated that KSR1 is, indeed, a direct substrate of praja2 (Figure 16D).
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KSR1
Figure 16. praja2 ubiquitinates KSR1. (A) HEK293 cells were co-transfected with HA-ubiquitin, and praja2 or praja2rm. After 24 hours cells were harvested and the lysates were immunoprecipitated with anti-KSR1 and immunoblotted with anti-HA, anti-KSR1 and anti-flag antibodies. (B) HEK293 cells were transfected with HA-tagged ubiquitin. 24 hours after transfection, cells were either left untreated or stimulated with EGF (100ng/ml) at indicated time. Lysates were immunoprecipitated with anti-KSR1 and immunoblotted with anti-HA, anti-KSR1 antibodies. (C) HEK293 were co-transfected as in Figure (A) and stimulated at indicated time with EGF. (D) In vitro translated, $^{35}$S-labeled KSR1 was incubated with his6-tagged ubiquitin, in the presence or absence of E1, UbcH5c (E2) and anti-flag precipitates from HEK293 extract transiently transfected with praja2-flag, praja2rm. The reaction mixture was denatured, size-fractionated on 7% SDS-PAGE, and analyzed by autoradiography. A fraction of the reaction mixture was immunoblotted with anti-flag antibody (lower panel).
2.8 praja2 controls ERK1/2 signaling

The findings indicate that KSR1 in response to receptor activation becomes a relevant target of the ubiquitin pathway. Given the relevant role of KSR1 in ERK1/2 signalling, we investigated if praja2 modulates RTK-dependent ERK cascade. To this end, we monitored ERK1/2 phosphorylation at its active site (Thr202/Tyr204) in cells stimulated with EGF. As shown in Figures 17A and 17B, in control cells, EGF treatment induced ERK1/2 phosphorylation over time from stimulation. Expression of praja2 increased both basal and EGF-induced ERK phosphorylation, by several fold over control values. Furthermore, genetic knockdown of endogenous praja2 also sustained ERK1/2 phosphorylation, compared to controls (Figure 17C).
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Figure 17: praja2 control ERK1/2 signaling. (A) HEK293 or U2OS (B) cells were transfected with CMV or Flag-rm. 24 hours after transfection, cells were treated EGF (100ng/ml) for indicate times. Lysates were separated on SDS-page and immunoblotted with anti p-ERK antibody. Anti-flag antibody is used as transfection control and anti-ERK2 as loading control. (C) HEK293 cells were transfected with control siRNA or praja2 siRNA. Than they are processed as above. (D) Cumulative data of the experiment shown in A&B. Data are expressed as a mean value ± S.E.M of three independent experiments.
Next, we explored the functional relevance of the praja2 binding to KSR1 in ERK signalling. Cells were pre-treated for 6h with a stearated synthetic peptide spanning the praja2 binding motif of KSR1 (KRS1pep-flag) or with a flag-tagged scrambled peptide (CNTpep-flag). As shown in Figure 18, both peptides efficiently accumulated within the treated cells. The cells were then stimulated with EGF. ERK phosphorylation was monitored over a time-point curve. As shown in Figures 19, pre-treatment with KSR1pep-flag enhanced both, basal and EGF-induced ERK phosphorylation. The effects of KSR1pep on ERK signalling were replicated in other cell lines (HEK293 and U-87 MG), suggesting a more general role of praja2 in the control of KSR1 pathway.
Figure 18. The synthetic peptides accumulate efficiently within the cell. HEK293 cells were treated for 8 hours with a stearated synthetic peptide containing a Flag-tag spanning the praJA2 binding motif of KSR1 (Ksr1pep-flag) or with a flag-tagged scrambled peptide (CNTpep-flag). After cells are subjected to immunostaining with monoclonal anti-flag. Images were collected and analyzed by confocal microscopy. DRAQ5 staining for nuclei detection was used (blu).
Figure 19: the binding between praja2 and KSR1 is fundamental for ERK1\2 signalling. U2OS cells were pre-treated with CNTpep-flag or Ksr1pep-flag. After cells were treated with EGF (100ng/ml) for indicate times. Lysates were separated on SDS-page and immunoblotted with anti p-ERK antibody. Anti-flag antibody is used as transfection control and anti-ERK2 as loading control. (B) Cumulative data of the experiment shown in (A). Data are expressed as a mean value ± S.E.M of four independent experiments.
DISCUSSION
AND
CONCLUSION
Here, I report for the first time a new regulatory mechanism of KSR1 scaffold through ubiquitination. I found that, in course of growth factors stimulation, KSR1 undergoes to rapid poly-ubiquitination, which eventually leads to attenuation of the ERK pathway. Ubiquitination of KSR1 was induced by EGF. I identified praja2 as the principal E3 ligase responsible of KSR1 ubiquitination.

Signals originated at cell membrane are efficiently transmitted to downstream effectors and rapidly attenuated, eventually leading to resensitization of the pathway. The intricate interplay between ON/OFF state of the signaling pathway gives rise to signaling waves that rapidly propagate throughout the cell, eliciting biological responses that strictly depend on the intensity and frequency of the oscillatory circuits.

In this context KSR1 plays a key role as scaffold protein involved in the propagation and regulation of the mitogenic pathway of MAP kinases.

KSR1 contribution to the regulation of the MAPK signaling is finely tuned because of an auto-regulatory series of phosphorylation/dephosphorylation events on KSR1.

Indeed, phosphorylation on KSR1 by C-TAK1, in resting condition, keeps the scaffold inactive, until a growth stimulus activates PP2A (Muller j. et al. 2001; Roy F. et al. 2002; Ory S. et al. 2003). Dephosphorylation of KSR1 by PP2A activates the protein that, in turn, amplifies the MAPK cascade. When the signaling has to be turned off, activated ERK1/2 phosphorylates another site on KSR1, causing the disassembly of the multikinase complex and regulating the temporal duration of the signal. (McKay et al. 2009).

The principal mechanism regulating KSR1 activity was mostly based on phosphorylation. A variety of attenuation mechanisms have been identified, so far (Alexander Y. et al. 2008; Sandra L Harris and Arnold J Levine 2005; Errede B. 1996; Dhillon AS. 2007). In the case of ERK pathway, a negative loop between ERK and KSR1 complex ensures a cycle of activation/de-activation process that limits uncontrolled mitogenic signaling. Phosphorylation of KSR1 and B-Raf by locally activated ERK dissociates the KSR1 multi-kinase complex, turning-off the ERK pathway (McKay et al. 2009). Bi-directional regulation of KSR1 and ERK activity controls the rate, the magnitude and the persistence of downstream mitogenic pathways.

My work added a novel twist in the ERK cascade, identifying KSR1 as a target of the ubiquitin pathway and further exploring
the regulation of MAPK signal amplitude.

Ubiquitination of KSR1 was rapidly induced by EGF pathway, indicating that there is an even more tight step of regulation between the activation of KSR1 and the inactivation and disassembly of the complex. This paralleled downregulation of ERK1/2 cascade. Interference of praja2 expression/activity or expression of a praja2 mutant carrying mutations within H2-RING domain sites, negatively impacted on KSR1 ubiquitination, enhancing ERK activation and prolonging the wave of downstream signaling. A peptide targeting the praja2 binding interface on KSR1 by preventing Praja2 binding to KSR1 and consequent ubiquitination of the scaffold increased both, basal and ligand-induced ERK phosphorylation. These findings further support the role of praja2 in the negative regulation of KSR1 signaling.

Hence, praja2 acts in response to EGF stimulation in promoting KSR1 ubiquitination and ERK attenuation, pointing to a more general role of praja2 in controlling of the strength and duration of KSR1-ERK cascade (Figure 20).
Figure 20. praja2-KSR model. Stimulation of receptors by a ligand that activates KSR1/2, which in turn induce sequential activation of mitogenic kinases (ON). Recruitment of praja2 within the multi-kinase scaffold induces poly-ubiquitination of KSR, and consequent attenuation of the ERK pathway (OFF).

In conclusion, the findings highlight the importance of the praja2-mediated ubiquitination of KSR1 in the control of mitogenic signaling. Exploring further the mechanism(s) regulating ubiquitination of KSR1 in course of growth factor stimulation, and identifying additional praja2 targets in the ERK1/2 cascade will provide essential tools to dissect and manipulate these important signaling pathways.
METHODS
Cell lines. Human embryonic kidney cell line (HEK293), glioblastoma cells (U87MG) and osteosarcoma cells (U2OS) were cultured in Dulbecco modified Eagle’s medium containing 10% fetal bovine serum in an atmosphere of 5% CO2.

Plasmids and transfection. Vectors encoding the flag-praja2 and GST-praja2 were purchased from Genecopeia. praja2rm was generated by site-directed mutagenesis, while praja2 deletion mutants and GST-fusions were generated by PCR with specific oligonucleotide primers. PCR products were sub-cloned into the same vector of wild-type praja2 cDNA. HA-tagged ubiquitin was provided by Dr Antonio Leonardi (University of Naples, Italy); flag-tagged KSR vectors was provided by Addgene Vector Database. GST-fusion peptides were generated by Eduard Stefan (University of Innsbruck); SMART pool siRNAs targeting coding regions were purchased from Dharmacon. The following are the siRNA sequences (Thermo Scientific) targeting human praja2: sequence 1: 5’GAAGCACCUCUUACCUUGA-3’; sequence 2: 5’AGACUGCUCUGGCCCAUUU-3’; sequence 3: 5’-GCAGGAGGUAUCAGACAA-3’; sequence 4: 5’-GUUAGAUUCUGUACCAUUA-3’.

The siRNAs were transiently transfected using Lipofectamine 2000 (Invitrogen) at a final concentration of 100 pmol/ml of culture medium.

Antibodies and chemicals. Rabbit polyclonal antibodies directed against KSR1 (immunoblot dilution 1:1000, immunostaining dilution 1:100) and Phospho44/42 MAPK (P-ERK1/2)(immunoblot dilution 1:1000) were bought from Cell Signaling; ERK2(immunoblot dilution 1:8000) and glutathione S-transferase (GST)(immunoblot dilution 1:8000) from Santa Cruz; α-tubulin (immunoblot dilution 1:8000), flag (immunoblot dilution 1:4000, immunostaining dilution 1:400, immunoprecipitation dilution 1:200 ) and myc epitope (immunoblot dilution 1:1000) from Sigma; hemaglutinin epitope (immunoblot dilution 1:1000) (HA.11) from Covance; Rabbit polyclonal antibodies directed against praja2 (immunoblot dilution 1:1000, immunostaining dilution 1:400) was purchased from Bethyl. Fluorescein- or rhodamine-tagged anti-rabbit and anti-mouse IgG secondary antibodies were purchased from Technogenetics. EGF (used 100ng/ml) were purchased from Sigma.
**Western Blot Analysis.** Cells were washed twice with phosphate-buffered saline and lysed in saline buffer-1% Triton-X 100 (NaCl, 150 mM; Tris-HCl, 50 mM, pH8; EDTA, 5 mM) or, for immunoprecipitation assay, in saline buffer 0.5% NP40 (50mMTris-HCl, pH 7.4, 0.15MNaCl, 100 mM EDTA, 0.5% NP40) containing aprotinin (5 μg/ml), leupeptin (10 μg/ml), pepstatin (2 μg/ml), 0.5 mM PMSF, 2 mM orthovanadate, and 10 mM NaF. The lysates were cleared by centrifugation at 15,000 g for 10 min. An aliquote of whole cell lysate (WHL) (100 μg) were resolved on sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred on nitrocellulose membrane (Biorad, Milan, Italy) for 3 h. Filters were blocked for 1 h at room temperature in Tween-20 Phosphate buffer saline (TPBS) (PBS- Sigma, 0.1% Tween 20, pH 7.4) containing 5% non-fat dry milk. Blots were then incubated O/N with primary antibody. Blots were washed three times with TPBS buffer and then incubated for 1 h with secondary antibody (peroxidase-coupled anti-rabbit (GE-Healthcare) in TPBS. Reactive signals were revealed by enhanced ECL Western Blotting analysis system (Roche).

**Immunoprecipitation and pull-down assay.** Cells were homogenized in lysis buffer described above. Cell lysates (1.5 mg) were immunoprecipitated with the indicated antibodies. An aliquot of cell lysate (100 μg) or immunoprecipitated were resolved by SDS-PAGE gel and transferred to Protran membrane. The immunoblot analysis was performed as previously described. GST fusions were expressed and purified from BL21 (DE3) pLysS cells. 20 μl of GST or GST-praja2 beads were incubated with 2 mg of cell lysate or with in vitro translated [35S]-labeled KSR1 in 200 μl lysis buffer (150 mMNaCl, 50 mMTris-HCl pH 7.5, 1 mM EDTA, 0.5% triton X-100) in rotation at 4 °C overnight. Pellets were washed four times in lysis buffer supplemented with NaCl (0.4 M final concentration) and eluted in Laemmli buffer. Eluted samples were resolved on 8%-SDS PAGE gel, transferred to nitrocellulose membranes and immunoblotted with the indicated antibody. Pull down between GST-control peptide or GST-KSR1 peptide and overexpressed flag-praja2 cells was performed as described above.

**In vitro ubiquitination assay.** [35S]-labeled KSR1 was synthesized in vitro using TnT quick coupled transcription/translation system (Promega) in the presence of 45μCi of [35S]-labeled methionine. The ubiquitination assay was performed in buffer containing 50 mM Tri-HCl pH 7.5, 0.6 Mmdithiothreitol, 5mM MgCl2, supplemented with recombinant his-ubiquitin (2.5 μg/μl), 2 mM
ATP, E1 (1.5 ng/µl) (Affinity Research, Exeter, UK), purified E2 (10 ng/µl) (UbcH5b) in the presence or absence of agarose beads-bound praja2-flag or its mutants immunoprecipitated from cell lysates. The reaction mixture was incubated at 30°C for 90 min, then stopped with Laemmli buffer and resolved on 8% SDS-PAGE. Ubiquitination products were visualized by autoradiography.

Confocal microscopy and image analysis. Cells were plated on poly-L-lysine (10µg/ml) coated glass cover slips. Growing or peptides-treated cells were rinsed with PBS and fixed in 3% paraformaldehyde for 20 min. After permeabilization with 0.5% Triton X-100 in PBS for 5 min, the cells were incubated with 1× PBS, 0.1 mg/ml bovine serum albumin for 60 min at room temperature. Double immunofluorescence was carried out with the following antibodies: anti-praja2 (1:400) and anti-KSR1 (1:100) or flag-tag (1:400) antibodies. Fluorescein- or rhodamine-tagged anti-rabbit and anti-mouse IgG (Technogenetics) secondary antibodies were used. Immunofluorescence was visualized using a Zeiss LSM 510 Meta argon/krypton laser scanning confocal microscope. Quantification of the immunofluorescent images and correlation (Pearson’s) coefficient were calculated by Image-J software.

KSR1-Peptides treatment. Growing or, were described, serum-deprived cells were treated with 1 µM of scramble peptide (cnt-pep) or KSR-peptide (KSR-pep) for 9 hours before stimulation.

The following are the peptides sequences:

scramble peptide (cnt-pep) =

stearic group-FLQWADESRPSLLMDYKD

KSR1 peptide (KSR-pep) =

stearic group-WAFDLQERPSLLMD

Statistics. All data are presented as mean ± SEM and “n” indicates the number of slices. Statistical significance was evaluated by unpaired Student’s t test. Statistical significance was set at p < 0.05.
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Proteolysis of MOB1 by the ubiquitin ligase praja2 attenuates Hippo signalling and supports glioblastoma growth

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Human glioblastoma is the most frequent and aggressive form of brain tumour in the adult population. Proteolytic turnover of tumour suppressors by the ubiquitin-proteasome system is a mechanism that tumour cells can adopt to sustain their growth and invasiveness. However, the identity of ubiquitin-proteasome targets and regulatory in glioblastoma are still unknown. Here we report that the RING ligase praja2 ubiquitylates and degrades Mob, a core component of NDR/LATS kinase and a positive regulator of the tumour-suppressor Hippo cascade. Degradation of Mob through the ubiquitin-proteasome system attenuates the Hippo cascade and sustains glioblastoma growth in vitro. Accordingly, accumulation of praja2 during the transition from low- to high-grade glioma is associated with significant downregulation of the Hippo pathway. These findings identify praja2 as a novel upstream regulator of the Hippo cascade, linking the ubiquitin-proteasome system to deregulated glioblastoma growth.
Glialomas are malignant brain tumours that arise from glial cells. They are the most common primary proliferative disorders of the central nervous system in the adult population. They include a heterogenous group of tumours that are characterized by a variety of differentiation and malignancy peaks (2). Glioblastoma multiforme (GBM), which represents about 50% of total gliomas, is the most aggressive form of brain tumours, with a very poor median survival after initial diagnosis (3).

High-grade gliomas are thought to derive from low-grade variants. These variants undergo sequential genetic alterations, giving rise to cell populations with more invasive behaviour, greater activation of mitogenic pathways, and loss of cell cycle control. Transition from low-to high-grade gliomas is also marked by activation of the hypoxia pathway, which promotes further genomic instability, cellular evolution and concomitant tumour expansion (4-13).

At the molecular level, GBMs are characterized by a wide array of genetic and epigenetic alterations. This heterogenic role of these abnormalities in glioma development has been functionally confirmed by in vivo studies. Thus, mutations or tumour suppressor genes (TSGs), constitutive activation of membrane receptors (EGFR), induction of presynaptic pathways (BRAF/MEK), aberrant gene transcription and metabolic dysfunction have all been linked mechanistically to the development and progression of GBMs (14-17). These abnormalities underlie the high recurrence rate and resistance of these tumours to current therapies. Although major advances have been made recently in identifying the relevant players that regulate glioma growth and development, so far the key mechanisms and the genes responsible for glioblastoma neoplastic transformation remain subjects of active investigation.

The ubiquitin-proteasome system (UPS) is emerging as an important control mechanism of cell metabolism, growth and survival. The UPS is the principal pathway for eliminating unneeded or damaged proteins (18). UPS complex ubiquitination of a target protein to its proteolytic cleavage. Ubiquitylation is a multistep process that involves the sequential action of three enzymes: activating enzymes (E1), conjugating enzymes (UBC, or EC2), and ligases (E3). E3-ubiquitin ligase genes fall into two main classes characterized by a HECT domain or a RING domain (20,21). HECT domain E3 ligases act catalytically to form a triazine intermediate in which ubiquitin is ligated to substrate with the RING domain. E3 ligases serve as a scaffold that brings together the substrate and the E2 ligase. p53 functionally relies on the stability of intracellular substrates and has a significant role in critical aspects of cellular stress responses (22).

During CAMP-stimulating G-protein-mediated signalling, we applied a protein-fragment complementation assay (PFA) protocol, which relies on the fragmented fragments of the YFP reporter protein into proximity, allowing for folding and reconstitution of the fluorescent reporter protein signal in vivo, similar to the intracellular nuclear localization of the positive control for the protein-protein interaction of a bicistronic dimer (5). This assay is independent of a continuous stimulus, allowing for folding and reconstitution of the fluorescent reporter protein signal in vivo, similar to the intracellular nuclear localization of the positive control for the protein-protein interaction of a bicistronic dimer (5). This assay is independent of a continuous stimulus, allowing for folding and reconstitution of the fluorescent reporter protein signal in vivo, similar to the intracellular nuclear localization of the positive control for the protein-protein interaction of a bicistronic dimer (5). This assay is independent of a continuous stimulus, allowing for folding and reconstitution of the fluorescent reporter protein signal in vivo, similar to the intracellular nuclear localization of the positive control for the protein-protein interaction of a bicistronic dimer (5).

To map the binary interaction sites, we performed a peptide screen using GST fusion proteins as bait and second to determine specific amino acids required for p53 binding to MOB1. Overlapping peptides derived from MOB1 and 'MOB1' were expressed as GST-fused fusion proteins as previously described (23). We identified one potential binding site located at the binding motif HKRIL in the C-terminal region of p53 (Fig. 1).

To test if the C-terminal region of p53 binds specifically to MOB1, we performed a peptide screen using GST-fused fusion proteins as bait and second to determine specific amino acids required for p53 binding to MOB1. Overlapping peptides derived from MOB1 and 'MOB1' were expressed as GST-fused fusion proteins as previously described (23). We identified one potential binding site located at the binding motif HKRIL in the C-terminal region of p53 (Fig. 1).

Results: p53 interacts with Hippo pathway component MOB1. To gain insight into p53 function, we performed a yeast two-hybrid screening using a human brain cDNA library and the C-terminal domain of p53 as bait. Two independent clones encoding the C-terminal domain of p53 and an amino-terminal fragment of p53 (p53ΔN1-530) were isolated. p53ΔN1-530 is a core component of the nucleo-TdrER-related kinase 1 (NDRI)/LATS (large tumour suppressor) kinase complex that acts as a downstream effector of the Hippo pathway. Hippo is an evolutionally conserved protein kinase cascade that has a fundamental role in the control of cell growth and organ size (24).

First, we set out to verify the direct interaction of MOB1 and p53 in the two-hybrid system. We confirmed that p53 directly binds to MOB1 in vitro. A fusion protein carrying full-length p53ΔN1-530 fused to the C-terminal domain of a GST-fused fusion protein was isolated. GST-p53ΔN1-530 formed a stable complex (Fig. 1a). As p53 promotes degradation of MOB1 and protein, we treated the cells for 24 h with the proteasome inhibitor MG132 before harvesting in order to see the complex. Deletion mutagenesis and binding assays identified residues 637-638 as the p53 domain that interacts with MOB1 (Fig. 1a). Next, we demonstrated this interaction in vivo by isolating endogenous p53/MOB1 complex from cell lysates. LAT51 kinase was also present in the p53 MOB1 precipitates (Fig. 1b). As p53 promotes degradation of MOB1, we treated the cells for 24 h with the protease inhibitor MG132 before harvesting in order to see the complex. Deletion mutagenesis and binding assays identified residues 637-638 as the p53 domain that interacts with MOB1 (Fig. 1a).

Next, we demonstrated this interaction in vivo by isolating endogenous p53/MOB1 complex from cell lysates. LAT51 kinase was also present in the p53 precipitates (Fig. 1b). As p53 promotes degradation of p53, we treated the cells for 24 h with the protease inhibitor MG132 before harvesting in order to see the complex. Deletion mutagenesis and binding assays identified residues 637-638 as the p53 domain that interacts with MOB1 (Fig. 1a).

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Figure 1 | proap2 forms a complex with MOB1. (a) Schematic representation of the proap2 constructs used (upper panel). HEK293 cells were transiently transfected with HA-MOB1A and flag-tagged proap2 (either wildtype or deletion mutants). Cells were treated for 12 h with MG232 (10μM) before harvesting. Twenty-four hours following transfection, cells were harvested and lysed. Lysates were subjected to immunoprecipitation with anti-HA antibody. Precipitates were immunoblotted with anti-HA and flag antibodies (lower panel). (b) Endogenous proap2/MOB1A/LATS1 complex was isolated from cell lysates using anti-proap2 antibody. (c) In vitro-translated [35S]-labelled MOB1A and LATS1 were subjected to pull-down assays with purified GST or GST-proap2 fusion proteins (50 μg) followed by immunoblotting with anti-GST. The bar graph illustrates the densitometric quantitation of the average of n = 2 dot blot experiments. Dotted bars indicate the standard deviations. (d) Peptide libraries (20 μg) of MOB1A were covalently linked to recombinant 6×His-proap2 followed by immunoblotting with anti-GST. The bar graph illustrates the densitometric quantitation of the average of n = 2 dot blot experiments. Dotted bars indicate the standard deviations. (e) Heterogeneous expression of proap2/MOB1A/LATS1 complex in a single cell (upper panel). Scale bar, 10 μm. (f) U87MG cells were subjected to double immunostaining with monoclonal anti-MOB1 and polyclonal anti-proap2 antibodies. Images were captured and analyzed by confocal microscopy. Magnification of selected areas is shown (insets). Scale bar, 10 μm.
in the cytoplasm (Fig. 1f), and also at centrosomal region as shown by triple labelling with anti-MOB1, anti-praja2 and anti-γ-tubulin antibody (Supplementary Fig. 5a). A similar immunostaining pattern was also observed in FNP8, a primary culture of human GBM (Supplementary Fig. 5b).

praja2 ubiquititates and degrades MOB1. We postulated that praja2 ubiquititation promotes MOB1 degradation. We tested this hypothesis by measuring MOB1 levels in cells co-transfected with expression vectors encoding for flag-praja2 (either wild-type or RING mutant) and HA-tagged MOB1. We used two highly conserved variants of MOB1, MOB1-A and MOB1-B. As shown in Fig. 2a,b, expression of wild-type praja2 significantly reduced HA-MOB1 levels relative to the CMV vector control. In contrast, transfection with the inactive praja2 mutant (praja2zm) increased the concentration of HA-MOB1. Similar results were obtained with co-transfected MOB1 (Fig. 2d). Endogenous MOB1 was also efficiently degraded by transfected praja2 (Fig. 2e). Treatment with MG132 reversed the reduction by praja2 of the levels of HA-MOB1 (Fig. 2f) or of endogenous MOB1 (Fig. 2g). To demonstrate that praja2 affects MOB1 stability, rather than MOB1 synthesis, the experiments were repeated in presence of cycloheximide, an inhibitor of protein synthesis. Figure 2h) shows that MOB1 levels declined over time in control cells, with a half-life of ~5h. Knocking down endogenous praja2 significantly increased the half-life of endogenous MOB1 (Fig. 2h), whereas endogenous MOB1 (Supplementary Fig. 5c) or HA-MOB1 (Fig. 2h) were stabilized by praja2zm overexpression.

Since praja2 is an E3 ubiquitin ligase, we asked if praja2 ubiquititates MOB1 in living cells. Indeed, flag-praja2 promoted accumulation of poly-ubiquitylated forms of endogenous MOB1 (Fig. 2i) and of the co-transfected HA-MOB1 (Fig. 2j). Ligase activity was required for HA-MOB1 ubiquititation: expression of praja2zm did not enhance MOB1 modification (Fig. 2k). An in vivo ubiquititation assay confirmed that MOB1 is, in fact, a direct substrate of praja2 (Fig. 2a).

praja2 negatively regulates the Hippo pathway. YAP nuclear factor drives transcription of several genes. These include cyclin E and cdc25C, which induce quiescent cells to enter the cell cycle11. Upon reaching cell confluence and/or defined organ size, activated Hippo/MST phosphorylates the MOB1/LATS dimer, which leads to the activation of MOB1/LATS (ref. 32). Active MOB1/LATS complex phosphorylates YAP transcription factor at S127 (ref. 32). Phosphorylated YAP is trapped within the cytoplasm via interaction with 14-3-3 proteins and eventually degraded. Cytosolic sequestration of phosphorylated YAP suppresses the proliferating and antiapoptotic program, leading to cell cycle arrest and inhibition of organ or tumour growth33,34,35.

We postulated that praja2, by reducing MOB1 levels, would downregulate LATS activity, decreasing YAP phosphorylation and negatively impacting on the downstream Hippo cascade. We tested this idea by monitoring YAP phosphorylation at S127 (pYAP) and MOB1 levels in growing and in quiescent 878MG glioblastoma cells. As control cells reached confluence, YAP phosphorylation increased ~1.7-fold over baseline levels (Fig. 3a,b). Downregulation of endogenous praja2 (praja2 small interfering RNA (siRNA)) increased the levels of MOB1 and correspondingly enhanced YAP phosphorylation above control siRNA values (Fig. 3a,b). This was associated with marked reduction of YAP accumulation in the nuclei of praja2 siRNA-transfected cells (Fig. 3c,d). Direct interference with endogenous praja2 activity reproduced the effects of praja2 silencing. Thus, expression of the praja2zm mutant increased the levels of cyclin E and decreased cell proliferation in vitro36. As expected, silencing of praja2 significantly reduced cyclin E expression, both in U87MG cells and in primary cultures of human GBM (FNP8) (Fig. 3e).

praja2 is required for cell proliferation and tumour growth in vivo. The finding that praja2 downregulates the Hippo pathway provided a starting point to analyse how praja2 contributes to the phenotype of GBM cells. Hippo pathway activity is negatively correlated with growth and aggressive phenotype of GBM37. We predicted, therefore, that praja2 inactivation of MOB1 would enhance glioma cell transformation. First, we compared praja2 expression in FNP8 and in U87MG cells. Supplementary Figure S5a shows that praja2 is expressed at comparable levels in both cell types. To link causally praja2 to glioma cell malignancy, we asked how praja2 silencing affected cell growth. siRNA duplexes targeting human praja2 efficiently knocked-down endogenous protein (Supplementary Figure S5a). Importantly, downregulation of praja2 markedly inhibited growth of both U87MG (Fig. 3g) and FNP8 (Supplementary Fig. S5b) cells. The requirement of praja2 for cell growth was confirmed by fluorescence-activated cell sorter analysis. Thus, praja2 siRNA-transfected U87MG cells accumulated at the G1 phase of cell cycle (Fig. 3h). A reduction at G2-M transition was also evident in the praja2-silenced cells. Taken together, these findings indicate that praja2 supports the malignant GBM phenotype.

We further tested this notion in vivo using a mouse model of glioma tumorigenesis. In this model, U87MG cells are injected subcutaneously into C57 mice. The cells start to grow at the site of injection several days post-implantation38. To prove that praja2 is, in fact, required for tumour growth in vivo, we silenced endogenous praja2 in U87MG cells before injection by transient transfection with siRNA duplexes targeting praja2 (praja2 siRNA) or with control non-targeting siRNAs (control siRNA). Three weeks post-injection, the mice were killed and the weight of the lesions was scored. As shown in Fig. 4a, downregulation of praja2 significantly inhibited tumour growth. Similar results were obtained using primary cultures of human GBM (NMDP7) (Supplementary Fig. S6). We also proved the requirement for praja2 in vivo by a multiparameter and predictive rationale strategy (Supplementary Fig. S6). Treatment with siRNA targeting praja2 was transduced into glioblastoma xenografts grown in the same syngeneic mice. The levels of Ki67-positive cells was reduced by two-fold in tumours derived from praja2 siRNA-transfected cells compared to control siRNA-transfected cells (Fig. 4b). The levels of the cyclin-dependent kinase inhibitor p21, a molecular marker of G1 cell arrest, were significantly higher in praja2 siRNA-treated tumours, compared with controls (Fig. 4c,d), confirming the requirement of praja2 for cell cycle progression.

To study the role of praja2 in tumour development in a more physiological tissue context, we used an orthotopic glioblastoma model. U87MG cells transiently transfected with siRNAs (praja2 siRNA or control siRNA) were implanted stereotactically into the left caudate nucleus of mouse brain. The mice were killed 3 weeks later. Histological analysis of post-mortem control mouse brain revealed an homogeneous tumour mass with sharp borders that was clearly delimited from the adjacent normal brain tissue. The orthotopic tumour, like the subcutaneous tumour, was comprised mostly of large pleomorphic cells with abundant eosinophilic cytoplasm (Fig. 4e), and a dense tumour mass with clear borders. In contrast, in the tumours derived from praja2 siRNA-transfected mice, the tumours were predominantly comprised of small round cells with minimal or no cytoplasm (Fig. 4f). The frequency of Ki67-positive cells was significantly reduced in praja2 siRNA-transfected tumours, compared with controls (Fig. 4g), confirming the requirement of praja2 for cell cycle progression.
Figure 2 | proj2 ubiquitylates and degrades MOB1. (a–d) 293 cells were transiently co-transfected with CMV or flag proj2 (either wild type or RING mutant) and HA-MOB1 (a) or HA-MOB18 (c) vectors. Lysates were immunoblotted with the indicated antibodies. Quantitative analysis (mean ± s.e.m.) is reported in the panels (b, d), respectively. *P < 0.05 versus CMV; **P < 0.01 versus flag-proj2. (e) Immunoblot analysis of endogenous MOB1 on lysates from cells transiently transfected with CMV, flag-proj2 or flag-proj2M vector. Tubulin was used as a loading control. (f) Cells were transiently co-transfected with CMV or flag-proj2 and HA-MOB1. Lysates were immunoblotted with the indicated antibodies. The cells were treated for 4 h with MG132 (20 µM) before harvesting. (g) Cells were transiently transfected with CMV or flag-proj2 (either wild type or RING mutant) and treated for 8 h with MG132 (20 µM) before harvesting. Lysates were co-immunoblotted with the indicated antibodies. (h) Cells transiently transfected with control siRNA or flag-proj2 siRNA were treated with cycloheximide and harvested at the indicated time points after treatment. Lysates were immunoblotted with the indicated antibodies. (i) Cumulative analysis (mean ± s.e.m.) of the experiments shown in k. (j) Cells transiently co-transfected with NAU or NAU/ PROJECT and with either the expression vector for proj2 or proj3 were harvested. Lysates were co-immunoblotted with the indicated antibodies. (k) Cumulative analysis of the experiments shown in j. The data are expressed as mean ± s.e.m. of three independent experiments. (l) Cells were transiently co-transfected with NAU Ubicolly and flag-proj2 or flag-proj2M. Cells were treated for 8 h with MG132 (20 µM) before harvesting. Lysates were co-immunoprecipitated with anti-MOB1 antibody. The precipitates and lysates were immunoblotted with anti-MOB1 or anti-myc and anti-flag antibodies. (m) Same as in l, except cells were transiently co-transfected with HA-MOB1 and lysates were immunoprecipitated with anti-flag antibody. Precipitates and lysates were immunoblotted with anti-HA, anti-myc and anti-flag antibodies. (n) In (l), transfected (125I)-labelled MOB1 was incubated with the control or flag-proj2M immunoprecipitated from transfected cells, and visualised by autoradiography. A fraction of the reaction mixture was immunoblotted with anti-flag antibody (lower panel).
reduction of Ki67 immunoreactive signal in these tumours further supports the role of praj2. In regulating GliOM cellular proliferation (Fig. 4e). Growth of GliOM lesions in living mice was monitored by position emission tomography (PET) using the radiolabeled thymidine analogue \(^{18}F\)-fluorothymidine (\(^{18}F\)-FLT). \(^{18}F\)-PET-PET analysis was routinely used in vivo to trace cell proliferation in a milieu of intact tumour tissue\(^{11}\). FLT-PET scanning analysis revealed a two-fold reduction of \(^{18}F\)-FLT uptake by tumours derived from praj2 shRNA-transfected cells compared with controls (Fig. 4g,h). These findings provide further evidence that praj2 enhances glioma growth in vivo.

The praj2-MORI axis controls glioma growth in vivo. The data presented above indicate that accumulation of praj2 is positively correlated with glioma growth in vitro and in vivo. As MORI is a praj2 substrate, we hypothesized that downregulation of MORI by praj2 inhibits the Hippo pathway, thus enhancing GliOM growth. This hypothesis is supported by several additional findings. First, knock-down of praj2 significantly upregulated MORI in transplanted U87MG tumours (Fig. 5a and b). Second, concomitant downregulation of MORI restored growth of praj2-silenced glioblastoma cells (Fig. 5c). Downregulation of praj2 activated the Hippo pathway, as shown by increased autophosphorylation of LATS1 at its activation site serine 909 (p-LATS1).
Figure 4 | paja2 is required for tumour growth in vivo. (a) U87MG cells transiently transfected with siRNAs were subcutaneously implanted into CDI nude mice. Four weeks later the mice were killed and the tumours excised and weighed. Tumour sections were fixed and stained with hematoxylin/eosin or subjected to immunohistochemistry with anti-Ki-67 antibody. Scale bar, 20 μm. (b) Cumulative data of tumour weight are expressed as mean ± s.e.m. Twenty mice for each experimental group were used. *P < 0.01 versus control siRNA. Student’s t-test. (c) Expression of mTOR levels in tumour lysates are expressed as mean ± s.e.m. of three independent experiments. *P < 0.01 versus control siRNA. Student’s t-test. (d) U87MG cells transiently transfected with control siRNA or paja2 siRNA were stereotaxically implanted into the brain (left caudate nucleus) of nude mice. Tissue sections from tumour lesions were stained with hematoxylin/eosin or immunostained with anti-Ki-67. Scale bar, 400 μm. (e) Quantitative analysis of the paja2 levels in tumour lysates. Data are expressed as mean ± s.e.m. (Control siRNA: n = 15, paja2 siRNA: n = 10). *P < 0.05 versus control siRNA, Student’s t-test. (f) FLT-PET analysis was performed at 2 and 4 weeks post-implantation. (h) Quantitative analysis of the experiment (at 2 weeks) shown in g. Data are expressed as mean value ± s.e.m. *P < 0.05 versus control siRNA, Student’s t-test.
Figure 5 | The praj2-MOBI pathway controls tumor growth. (a) Western blot analysis for MOBI1 in cell lysates of tumor xenografts derived from mice inoculated with sRNA-transfected U87MG cells. (b) Quantitative analysis of the experiments shown in (a). Results are presented as a mean value ± s.e.m.; *p < 0.01 versus control siRNA. Student’s t-test. (c) Growth curve of U87MG siRNA-transfected cells. Following transfection, cells were seeded in 96-well plates, harvested at the indicated time points, and counted. A mean value ± s.e.m. is shown. (d) U87MG cells were transfected with the indicated sRNA. Lysates were immunoblotted with the indicated antibodies. (e) Quantitative analysis of autophosphorylation of LAT1 shown in (d). *p < 0.01 versus control siRNA. Student’s t-test. (f) U87MG cells transiently transfected with control siRNA, praj2 siRNA or praj2 siRNA and MOBI1 siRNA were stereotactically implanted into the brain (left caudate nucleus) of C57BL/6 mice. Tissue sections from tumor lesions were stained with hematoxylin/eosin. Scale bar: 400 μm (upper) and 20 μm (lower). (g) Cumulative analysis of tumors shown in (f). Number of animals tested: control siRNA: n = 5; praj2 siRNA: n = 5; praj2 siRNA + MOBI1 siRNA: n = 6. *p < 0.01 versus control siRNA and praj2 siRNA/MOBI1 siRNA. Student’s t-test.

(Fig. 5c) and phosphorylation of YAP at S122 (Fig. 5d). This parallels a global downregulation of cyclins. The replication cyclins, cyclin E and cyclin D were downregulated 10-fold and 4.8-fold, respectively, in praj2-silenced cells. Some downregulation of cyclin B (X6-40β) was also observed (Fig. 5d). Consistent with our hypothesis, concomitant downregulation of MOBI1 partially reversed the increase in LAT1 and YAP1 phosphorylation induced by praj2 silencing (Fig. 5e). Simultaneous knockdown of MOBI1 partially restored expression in the praj2 knockdown cells, particularly in the case of cyclin E, whose expression increased 5.9-fold. These data along with those reported in Fig. 3 suggest that praj2 primarily regulates G1/S progression. Lastly, inhibition of the Hippo signaling by MOBI1 knockdown restored tumour growth of praj2-silenced glioblastoma cells in mouse brain (Fig. 5f).

praj2 is overexpressed in human glioma. Given its positive role in glioma cell proliferation and xenograft tumour growth, we investigated the expression profile of praj2 in human gliomas. Tissue samples obtained from first biopsies of patients that underwent brain surgery for low-grade astrocytoma or GBM were homogenized and the protein lysates were immunoblotted with anti-praj2 antibody. Low levels of praj2 were detected in lysates from human astrocytomas, whereas praj2 concentrations were two-fold to three-fold higher in lysates from GBM samples (Fig. 6a,b). Quantitative RT-PCR analysis demonstrated that praj2 mRNA was increased in tumor biopsies of GBM, compared with astrocytoma II (Fig. 6c). This was not a consequence of amplification of the praj2 gene in GBM lesions, as shown by fluorescence in situ hybridization (Supplementary Fig. S7a,b). Next, we performed immunohistochemistry on whole sections.
Figure 6 | praja2 is overexpressed in high-grade human glioma. (a) lysates from tissue samples of astrocytoma (grade II) and GBM were immunoblotted with anti-praja2 antibody and anti-β-actin antibodies. (b) Cumulative analysis of praja2 levels in each category of tissue lysates. *P<0.01 versus astrocytoma II. Student’s t-test. (c) Expression analysis of praja2 mRNA by RT-qPCR. The graph shows the enhancement of praja2 transcript levels in astrocytoma II and GBM lesions. Levels of praja2 mRNA in normal human brain served as baseline. Statistically significant values at 0.001 by t-test analysis. Student’s t-test. (a) Tissue sections from human astrocytoma II and GBM were stained with hematoxylin and eosin (HE) or immunostained with anti-praja2 and anti-M0B1 antibody, and analysed by light microscopy. Scale bar = 25 μm. (e) GBM and astrocytoma tissue sections were immunostained with anti-YAP and anti-phospho-YAP antibody. Enlarged sections are shown on the right of each pair of images. Scale bar = 25 μm.

We then asked whether enhanced expression of praja2 in glioma tissues was associated with changes in the intracellular distribution of the ligase. Immunofluorescence analysis of glioma tissues is shown in Supplementary Fig. 5B. In astrocytoma tissue, moderate praja2 staining was homogeneously distributed at the cell periphery (Supplementary Fig. 5Ba,b). As expected, strong praja2 staining was observed in GBM cells (Supplementary Fig. 5Bc,d). This staining was diffusely distributed throughout the cytoplasm, with some concentration at the perinuclear membrane and the nuclear compartment. Conversely, the M0B1 immunofluorescent signal was intense in astrocytoma cells, whereas only a slight signal was detected in GBM lesions.
(Supplementary Fig S6A). Note that GBM sections contain heterogeneous cell populations marked by quite different levels of p300 and MOB1, possibly reflecting the high rate of genetic instability that defines these tumours.

Increased levels of p300 in GBM lesions were linked to significantly decreased YAP phosphorylation (Fig S6A). The unmodified YAP accumulated in the nuclear compartment (Fig S6A). In contrast, astrocytoma tissues present high levels of phosphorylated YAP, which is mostly distributed throughout the cytoplasm (Fig S6A).

**Discussion**

Here we report a mechanism of signal attenuation in human GBM based on a proteolytic turnover of components of an oncogenic signalling pathway. We find that p300 directly binds to and ubiquitylates MOB1, the regulatory component of the LATS1/2 kinases of the Hippo pathway. Degradation of ubiquitylated MOB1 by the proteasome inhibits this signalling cascade favoring GBM growth.

Proteolysis of cellular proteins by the UPS is a sophisticated mechanism controlling complex biological interactions that underpin cell survival, growth and metabolism. Accordingly, deregulation of the UPS is causally implicated in a wide variety of human diseases, including neurodegeneration and cancer. Modulating intracellular UPS activity is a therapeutic strategy currently being tested for treatment of several human disorders.

However, non-specific side effects and toxicity problems limit its use. An alternative approach, targeting the levels or activity of specific UPS ligases, is an attractive and, perhaps, more effective therapeutic strategy. The E3 ubiquitin ligase MDM2 represents the prototype target of inhibitors that selectively interfere with the ubiquitylation processes in human tumours. MDM2 is a transcriptional target of p53 and tightly regulates p53 levels by masking p53 for UPS degradation. This constitutes a negative feedback loop that controls the p53-dependent stress response. Decreased levels of p53 are found in many human cancers, resulting from genetic inactivation, viral infections or amplification of the MDM2 gene. Preventing MDM2-p53 interaction with small-molecule drugs restores normal p53 activity. This therapy is currently being tested in haematological and solid malignancies.

Changes in the expression profile of components of the ubiquitin ligase complexes have been reported in diverse tumour types. The H11, an ah-repressing enzyme that regulates cell cycle progression, is upregulated in high-grade astrocytomas. Conversely, the tumour suppressor F-box protein Fbw7, a component of the Nup133-F-box E3-ubiquitin ligase complex, is downregulated in high-grade gliomas, and serves as prognostic indicator for survival in glioma patients. Fbw7 mutations of ubiquitin ligases have been identified in GBM and in other human malignancies, further supporting a pathogenic role of deregulated UPS systems in brain tumours.

Our findings identify p300 as a novel cancer-associated gene whose expression is upregulated in high-grade gliomas. The expression profile of p300 in tissue biopsies of astrocytoma and GBM lesions is consistent with a direct correlation between the levels of the ligase and the malignant phenotype of the tumour.

At the mechanistic level, we identified MOB1, the regulatory component of the LATS1/2 kinases, as a crucial p300 substrate. MOB1 family members (MOB1A and MOB1B) are products of highly conserved genes originally identified as regulators of mitotic exit and cytokinesis in yeast, and later reported as tumour suppressors and components of the Hippo/NDR/LATS1/2 pathways. MOB1 interacts with and activates LATS kinases. The MOB1/LATS1 kinase complex phosphorylates and inactivates YAP, a transcription factor that controls entry into the cell cycle of quiescent cells. Phosphorylated YAP is recruited and ubiquitylated by the SCF-Cbl-bcr(CD) E3-ubiquitin ligase complex, which inactivates YAP degraded through the proteasome. The Hippo pathway can also be attenuated by proteolytic turnover of LATS1/2 kinase, LATS1 is ubiquitylated by the FBR1 class E3-ubiquitin ligase TCE1, which interacts with the PPIase motif of LATS1 through its WW domains. Proteolysis...
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of ubiquitinated LATS1 enhances cell growth, induces epithelial-mesenchymal transition and increases tumorigenicity. In the work described above, we report that the regulatory component of the LATS1 kinase, MOBI, is degraded by p27 through the UPS. Proteolysis of MOBI by p27 attenuates the Hippo cascade and enhances proliferation of glioblastoma cells. As MOBI interacts with NDR1/2 kinases, this suggests that p27 may also impact on the NDR1/2-dependent signalling cascade. This mechanism operates in vivo, downregulating of MOBI restored the proliferative activity of p27-altered GBM cells and sustained tumour growth. By removing the inhibitor constraint imposed by Hippo, p27 would favour YAP-dependent gene transcription, coupling the UPS pathway to GBM growth (Fig. 7). Notably, we found that MOBI levels inversely correlate with p27 abundance and glioma malignancy, supporting the role of p27 in control of MOBI stability in vivo. Moreover, upregulation of p27 in GBM lesions is linked to significant inhibition of the Hippo pathway, confirming the mechanistic link between p27, Hippo pathway and GBM.

Our results, taken together, identify the E3 ligase p27 as a novel cancer-associated gene whose expression predicts the aggressive potential of glioma. Negative regulation of the Hippo pathway by p27 constitutes a UPS-driven signalling circuit that sustains growth of glial cells carrying various oncogenic mutations. Unveiling the mechanism(s) regulating p27 expression and activity in glioma cells and identifying additional relevant p27 targets will contribute to our understanding of the role of UPS pathway in the development and progression of human GBMs. We expect this to inform the design of more effective target-oriented therapeutic strategies for aggressive human GBMs.

Methods

Latin and tissue. The human embryonic kidney cell line (HEK-293) was cultured in DMEM containing 10% fetal bovine serum (FBS) in an atmosphere of 5% CO2. The glioblastoma cell line U87MG (grade IV) was purchased from the American Type Culture Collection and maintained in modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and 2 mM glutamine, penicillin and streptomycin. Cells were maintained in a humidified incubator at 37°C and 95% humidity. Primary glioblastoma cells (C782 and MBBP) were prepared from enucleated tissue samples surgically removed from untreated patients. All patient data were obtained from informed consent and were used to carry out glioblastoma multilineage (according to WHO-classification). All tumors were positive for vimentin, GFAP (glial fibrillary acidic protein) and EGR.

Transfection of plasmids and siRNAs. Vectors encoding for wild-type or mutant p27, ubiquitin, MOBI, and p27 were described previously.22,23 Transfection efficiency was monitored by including a GFP vector in the transfection mixture. SMARTpool siRNAs against p27 were used at a final concentration of 100 nM. RNA was isolated using TRIzol reagent (Invitrogen). Reverse transcription and qRT-PCR were performed using SYBR Green PCR master mix (Applied Biosystems). The following primers were used: F: 5'-AGACACTACCTAAGGGAGGTCA-3'; R: 5'-GACACCCCACTACGCCACATG-3'. The ABI PRISM 7000 Sequence Detection System (Applied Biosystems) was used for qRT-PCRs. The relative quantification of the expression levels was determined using the 2-ΔΔCt method. The following primers were used: F: 5'-AGACACTACCTAAGGGAGGTCA-3'; R: 5'-GACACCCCACTACGCCACATG-3'. The ABI PRISM 7000 Sequence Detection System (Applied Biosystems) was used for qRT-PCRs. The relative quantification of the expression levels was determined using the 2-ΔΔCt method.

Antibodies and chemicals. Goat and rabbit polyclonal antibodies directed against MOBI were purchased from SantaCruz and Cell Signalling, and used at working dilutions of 1:500 for immunofluorescence and 1:1000 for immunoblotting. Mouse anti-asialo GM1 (12/50) detection (1:100), Evodia (1:1000, Cell Signalling, #9725), and rabbit anti-GFP (1:1000, Cell Signalling, #4000) were purchased from Covance, rabbit anti-human, rabbit, and mouse, respectively. All antibodies were used at their recommended dilution. For siRNA treatment, H161 cells stably expressing scramble siRNA were transfected using Lipo процент (Invitrogen) at a final concentration of 100 nM. The cellular extracts were processed and run on SDS-PAGE and transferred to nitrocellulose membranes (Millipore). The membranes were probed with the secondary antibodies conjugated to horseradish peroxidase. Immunoreactivity was visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech) and quantitated using a charge-coupled device system.
reconstructed temporal and spatial states of the explore-105 navigator in the liver volumetric acquisition with multislice time of flight (MRA) with EPI readout, a technique that has been shown to be useful in the evaluation of liver tumors and lesions.

**Immunofluorescence and cell tracking.** Sections from human glioblastoma specimens were incubated with DAPI and primary antibodies. Immunofluorescence images were visualized using a Zeiss LSM 510 confocal laser scanning microscope. Images from each optical section were averaged to improve the signal-to-noise ratio. Images from a minimum of four sections per tumor type and at least four samples of each category of tumor were collected and analyzed. Immunofluorescence on cultured cells was performed as described previously.

**Fluorescence-activated cell sorting.** Tumor cell lysates and samples were mixed with propidium iodide (PI). Cells were fixed with 70% ethanol and nuclearly kept at 4°C overnight. Cells were washed twice with PBS rinsed in PBS containing 40 μg/mL propidium iodide (PI; Sigma) and 70 μg/mL RNase A (Promega). The cell viability of the stained samples was analyzed by flow cytometry using FACSCalibur (BD Biosciences) and CellQuest (BD Biosciences) software. Results are presented as mean ± SEM of three separate experiments.

**In vitro protein binding assays.** GII hybrid proteins were expressed in BHK-21 cells (cat. no. HE212). Induction cell lysates and affinity-purified hybrid proteins were performed as recommended by the supplier of the FabX vectors (GenScript). GII hybrid proteins (GII TATMAMV) expressed in BHK-21 cells were solubilized in 1% Igepal-C6.00 (Sigma), sonicated and centrifuged at 14,000 rpm for 10 minutes. The supernatant was collected and then subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. Blots were probed with rabbit polyclonal antibodies to GII (1:500 dilution) and then reacted with HRP-conjugated secondary antibodies. The membrane was developed with ECL Plus (Amersham Biosciences). Signals were quantified using a ChemiDoc XRS+ system (Bio-Rad).

**References.**


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Author contributions

L.L., E.S. and A.P. designed the experiments. L.L. carried out most of the experiments except the following: R.D.D., Fig. 1b, LB Fig. 1c, 3c, 3d, 2g, 2e, 2f, and V.A.G. Fig. 1d, 1f. Figs. 3a–d, 2a–c, 2d, 2f, Supplementary Fig. 3a–d, Supplementary Fig. 1b, 1c, 2c, 4c, 4d, 5a, 5b, 5c, 6a, 6b, 6c, 6d, 8a, 8b, 8c, 8d, 8e, and 8f were prepared using FlowJo software (FlowJo LLC, Oregon, USA).

Supplementary Information

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Proteolytic control of neurite outgrowth inhibitor NOGO-A by the cAMP/PKA pathway

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Protein kinase A (PKA) controls major aspects of neurite outgrowth and morphogenesis and plays an essential role in synaptic plasticity and memory. Therefore, the molecular mechanisms of PKA action on neurite spurring and activity are still unknown. Here, we report that in response to neurotranspherin or cAMP stimulation, the RING ligase praj2 ubiquitinates and degrades NOGO-A, a major inhibitor of neurite outgrowth in mammalian brain. Genetic ablation of praj2 severely inhibits neurite extension of differentiating neuroblastoma cells and neurite arborization and synapse formation in cortical terminals in developing rat brain. This phenotype was rescued when both praj2 and NOGO-A were deleted, suggesting that NOGO-A is, indeed, a biologically relevant target of praj2 in neuronal cells. Our findings unveil a novel mechanism that functionally couples cAMP signaling with the proteolytic turnover of NOGO-A, positively impacting on neurite outgrowth in mammalian brain.

cAMP | PKA | praj2 | ubiquitin | NOGO-A

Neurite outgrowth plays an essential role in embryonic development, neuronal differentiation, and central nervous system (CNS) plasticity. Outgrowth can be also altered in several neurological disorders, as well as by neuronal injury and degeneration (1, 2). Extracellular signals, such as neurotrophic factors (NTFs) and neurotransmitters, regulate neurite outgrowth, dendritic arborization, and synaptic activity, establishing a dynamic neuronal network in developing and adult CNS. NTFs and neurotransmitters act at the cell membrane by generating intracellular second messengers that, in turn, reversibly modulate the activity of signaling proteins and effectors enzymes (3, 4).

cAMP is an ancient second messenger that controls a variety of biological cues. In neurons, essential functions such as neurite outgrowth and morphogenesis, synaptic transmission, and plasticity require tightly regulated inositol 1,4,5-trisphosphate (IP3) signaling. AKAPs form a broad transduction unit, which contains A-kinase anchoring proteins (AKAPs) and IP3R2 (5). AKAPs allow the formation of a signaling complex, consisting of cyclase, PKA, and IP3R2, which is essential for the regulation of intracellular signaling pathways. AKAPs can also interact with the cAMP cascade (6, 7), and they transduce the intracellular signals to the extracellular environment. Space-proximate adhesion of FAKa to PKA promotes the activation of PKA and the accumulation of cAMP in the CNS. The molecular targets involved are unknown. NOGO-A is a member of the retinoic acid-inducible family of integral membrane proteins with a conserved C terminus that is essential for homodimerization and is expressed in oligodendrocytes.
Because the R14D motif is highly conserved among RTNs, we tested it first family members from a codon variant flag-pull2. Fig. S1 shows that RTNGC and RTN5C bind pull2, suggesting that binding to pull2 is a general feature of the RTN protein family.

In situ immunostaining of human neurodegeneration cells demonstrated that flag-pull2, but not its deletion mutant Flag-A551-358, partially colocalized with NGGCA-A. Overlapping signals were obtained in the perinuclear region, cytoplasm, and cell membrane, supporting the existence of a complex in intact cells (Fig. 1E).

Protocol of NOGO-A by pull2. In a typical in vitro experiment, NOGO-A was expressed in HEK293 cells using a plasmid encoding Flag-Nogo-A and a plasmid encoding pull2. Transfected cells were cultured for 48 h, then harvested for immunoprecipitation with anti-Flag and anti-pull2 antibodies (primary). The lysate was subjected to immunoprecipitation with anti-Flag and anti-Cterminus antibodies (primary). The precipitates were subjected to immunoblotting with anti-Flag antibodies (primary) and anti-Cterminus antibodies (secondary). The band was visualized using an Odyssey infrared imaging system.

Fig. 2. Pull2 enhances and regulates NOGO-A. A) Immunoblot of lysates from cells transiently transfected with vectors encoding Flag-pull2 (five different constructs) and Myc-NOGO-A. B) Quantitative analysis of the relative band intensities as compared to the band intensity of the control untreated cells. C) Immunoblot of NOGO-A expression levels in cells transfected with Flag-pull2 and treated with 10 nM of R14D motif. D) Immunoblot of NOGO-A expression levels in cells transfected with Flag-pull2 and treated with 10 nM of R14D motif. E) Immunoblot of NOGO-A expression levels in cells transfected with Flag-pull2 and treated with 10 nM of R14D motif. F) Immunoblot of NOGO-A expression levels in cells transfected with Flag-pull2 and treated with 10 nM of R14D motif. G) Immunoblot of NOGO-A expression levels in cells transfected with Flag-pull2 and treated with 10 nM of R14D motif. H) Immunoblot of NOGO-A expression levels in cells transfected with Flag-pull2 and treated with 10 nM of R14D motif. I) Immunoblot of NOGO-A expression levels in cells transfected with Flag-pull2 and treated with 10 nM of R14D motif. J) Immunoblot of NOGO-A expression levels in cells transfected with Flag-pull2 and treated with 10 nM of R14D motif.
**proja** is required for NOGO-A degradation and neurite outgrowth in differentiating neurons. In light of the inhibitory role of NOGO-A in neurite outgrowth of developing or injured neurons, we asked whether, by affecting NOGO-A stability, proja regulates neurite extension in immature neuronal cells. To this end, we used SH-SY5Y neuroblastoma cells as a model system. SH-SY5Y cells differentiate in vitro in the presence of retinoic acid (RA) and brain-derived neurotrophic factor (BDNF) (14). RA-treated cells exhibit a neuron-like morphology and express neuron-specific proteins. Addition of BDNF to the medium of RA-treated cells induces additional major morphological changes and promotes neurite formation. We reproduced this neuron-like phenotype by sequentially treating SH-SY5Y cells with RA and BDNF (Fig. 3 A). Genetic knockdown of proja using a mixture of two siRNAs targeting proja reduced the neuron-like phenotype of these cells (Fig. 3, A, B, and C). Similar effects were obtained using two independent siRNAs targeting distinct coding region (Fig. 3D) or the 3′ UTR of proja (14). Moreover, the neurite extension in siRNA-transfected cells could be rescued by co-expression of exogenous proja2 (Fig. 3, E and F). The relative abundance of NOGO-A and proja in transfected cells is shown (Fig. 3G).

The effects of proja2 downregulation on neurite extension might result from either impairment of cAMP signaling or the accumulation of NOGO-A. To discriminate between these two possibilities, we performed a double knockdown for proja2 and NOGO-A. Downregulation of NOGO-A restored, at least in part, neurite extension in proja2-silenced neuroblastoma cells (Fig. 3, C and D). These data were confirmed by immunostaining differentiating SH-SY5Y cells with an anti-actin antibody (Fig. 3B). Fig. 3D shows the levels of proja1 and proja2-mediated NOGO-A degradation and induced a time-dependent rise of exogenous (Fig. 2 C and D) and endogenous (Fig. 2 E and F and Fig. 3B) NOGO-A levels. Next, we investigated if proja2 promotes ubiquitination of NOGO-A. As expected, expression of proja2, but not proja2m, leads to accumulation of polyubiquitinated NOGO-A (Fig. 2D), proja2 activity is induced by FSK (9). Therefore, we tested if FSK induction regulated ubiquitination of NOGO-A. Cells were serum-starved overnight and subsequently treated with forskolin (FSK), an adenylate cyclase activator. As shown in Fig. 2H, raising intracellular cAMP levels positively impacted on NOGO-A ubiquitination, even at 3h from forskolin treatment. Expression of Flag-proja2m co-regulated both basal and FSK-stimulated NOGO-A ubiquitination, confirming the role of proja2 in the control of NOGO-A stability.
phosphorylation of pra2 at Ser342/Thr389 is required for ligase activity (9). Therefore, we monitored NOGO-A levels in cells expressing a pra2 mutant carrying substitution of Ser342/Thr389 to Ala (double mutant, pra2DM). As suspected, pra2DM failed to degrade compressed NOGO-A, compared with wild-type pra2 (Fig. 5C). When expressed in differentiating neuroblastoma cells, pra2DM prevented FSK-induced proteolysis of NOGO-A (Fig. 5D and E) and severely reduced neurite extension (Fig. 5F and G).

Next, we investigated the role of pra2 in mesencephalic neurons both in culture and in developing rat brain. As expected, pra2 silencing significantly reduced neurite extension of cultured mesencephalic neurons. The total length of neurites in pra2-aRNA-treated neurons was significantly lower compared with controls, as observed at three different time periods after transfection (Fig. 6A and B). Notably, after 6 d of silencing, the length of tyrosine hydroxylase (TH)-positive axons, clearly recognizable from the typical axonal varicocities, was reduced by 55.5% in pra2-aRNA-treated neurons (neurite lengths were

Fig. 5. pra2 mediates cAMP effects on neurite outgrowth. (A) Differentiating SH-SY5Y cells were transfected with the indicated siRNAs and treated with FSK (10 μM) and neurite lengths were scored 3 d later. (B) Cumulative data of three independent experiments as a mean value ± SEM are shown. *P < 0.05 versus xCTL. (C) Expression levels of Flag-pra2, Flag-DM, and NOGO-A (9) immunoblot analysis of lysates from SH-SY5Y cells transiently transfected with DM and pra2DM treated with chondrodysplasia (100 μg/mL) and forskolin (60 μM). (D) Cumulative data of three independent experiments are expressed as a mean value ± SEM. **P < 0.005 versus control [DM: ± chondrodysplasia (CCK) and Flag DM (CCK + FSK)]. (E) Differentiating SH-SY5Y cells cotransfected with pra2DM and GFP vector were immunostained with anti-Flag antibody and analyzed by confocal microscopy. (F) Cumulative data of three independent experiments as a mean value ± SEM are shown. *P < 0.05 versus GFP.

NOGO-A in aRNA-transfected cells. Although pra2 regulates RTN stability, no major morphological changes of the endoplasmic reticulum were observed following pra2 knockdown (Fig. 55). Moreover, in situ immunostaining analysis showed overlapping signals between NOGO-A and calbindin, whereas only a partial colocalization could be assigned to pra2 and calbindin (Fig. 56).

pra2 mediates cAMP effects on NOGO-A Stability and Neurite Outgrowth. Activation of the cAMP/PKA pathway positively regulates neurite outgrowth in a wide variety of species, profoundly impacting brain development and synaptic plasticity (15–17). Accordingly, treating neuroblastoma cells with forskolin or BDNF promoted neurite outgrowth and extension (Fig. 4A, b and c, and B). Silencing of pra2 prevented neurite extension induced by FSK and BDNF (Fig. 4A, B, and F and G), this phenotype was rescued when both pra2 and NOGO-A were depleted (Fig. 5A and B), suggesting that pra2 is, indeed, a relevant downstream effector of cAMP signaling on neuronal differentiation. The neurite extension induced by FSK was PKA-dependent, as treatment with the PKA inhibitor H89 reduced neurite elongation in both control and pra2-depleted cells (Fig. 5F).
The density of TH-positive varicosities in the dorsal striatum was assessed on average of six sections. To automatically delineate the fibers, the images were first thresholded and subsequently quantified with the Analyze Particles tool (ImageJ). Statistical analysis was performed using two-tailed Student t-test with Welch’s correction. *p < 0.05 versus siRNA, **p < 0.01 versus siRNA. Treatment with siRNA for 3 days led to a significant reduction in TH-positive varicosities compared to controls (Fig. 6A, B). The reduction in TH-positive varicosities was observed in the dorsal striatum, with no significant changes in the ventral striatum (Fig. 6C, D).

Discussion
Here, we demonstrate that cAMP promotes degradation of NOGO-A through the ubiquitin proteasome system. We identified pra2 as the E3 ubiquitin ligase that mediates cAMP-dependent degradation of NOGO-A. Disruption of NOGO-A by pra2 is a mechanism that underlies CAMP-induced neurite outgrowth in differentiating neural cells. Genetic downregulation of pra2 prevents NOGO-A degradation and inhibits axon outgrowth and branching in cultured neurons and in the nigrostriatal dopaminergic pathway of rat brain.

BDNF is essential for neuronal development, survival, and differentiation. Acting through a dedicated receptor at the cell membrane, BDNF stimulates a number of second messenger pathways that, in turn, activate distinct classes of adaptor, scaffold, and signaling enzymes. cAMP and its effector enzyme PKA have been implicated in mediating some of the effects of BDNF stimulation on neuronal differentiation and activity. BDNF stimulation of PI3K induces transient release of calcium from intracellular stores, activating calcium-sensitive adenylate cyclase and, consequently, elevation of cAMP levels (19, 20). Activation of PKA by cAMP increases the number and activity of neurite outgrowth, and neurite outgrowth in a variety of mammalian neurons (21, 22). Conversely, genetic or pharmacological inhibition of PKA severely downregulates neurite outgrowth and axonal extension, supporting the role of the cAMP/PKA pathway in critical aspects of neuronal differentiation (16). PrA phosphorylation of CREB and CREB-directed gene transcription represents the principal mechanism of cAMP action in promoting and sustaining neuronal differentiation (23). Additionally, a variety of intracellular substrates and downstream effectors of cAMP signaling have been identified and mechanistically linked to neuronal differentiation (24, 25).

Our results disclose a previously unrecognized mechanism of cAMP action on neuronal differentiation based on the regulated proteolysis of NOGO-A, a major inhibitor of neurite outgrowth in mammalian brain (26). cAMP induces proteolysis of NOGO-A through the ubiquitin proteasome system (UPS). Decrease of NOGO-A levels removes the inhibitor constraint on neurite outgrowth, preventing growth cone collapse and maintaining neurite extension and axon growth (16). We identified pra2 as the ubiquitin ligase responsible for ubiquitination and proteolysis of NOGO-A, pra2 is phosphorylated by the associated PKA, which stimulates ubiquitin ligase activity (5). Genetic knockdown of pra2 and pharmacological inhibition of PKA expression in NOGO-A-expressing neurons resulted in a significant reduction in neurite outgrowth and axonal extension (27).
of praj2 or expression of a phosphorylation-defective praj2 mutant prevented cAMP-induced NOGO-A degradation and severely inhibited neurite extension in differentiating neurons. Notably, downregulation of NOGO-A levels in praj2deficient cells restored neurite outgrowth to control values. Moreover, praj2 silencing was ineffective in downregulating neurite extension in NOGO-A KO neurons. Altogether, these findings are consistent with the notion that inhibition of neurite outgrowth by praj2 silencing depends on increased NOGO-A levels, rather than on global downregulation of cAMP signaling.

In conclusion, negative regulation of the NOGO-A pathway by cAMP and praj2 constitutes a novel UPS-driven signaling circuit that promotes and sustains biological processes underlying neuronal differentiation and synaptic activity. Uncovering the mechanism(s) regulating praj2 expression and activity in neurons and identifying relevant praj2 substrates operating in vivo will contribute to our understanding of the role of cAMP and UPS in the regulation of brain development and plasticity and may yield therapeutic strategies for neuropsychiatric disorders.

Materials and Methods

Cell Lines, HX239 and a neuroblastoma cell line SHSY-5Y were maintained in DMEM supplemented with 10% heat-inactivated FBS at 37°C, 5% (v/v) CO2, and 95% humidity. To induce differentiation, neuroblastoma cell lines were seeded at 5×10⁴–10⁵ cells per 60-mm dish (Sigma) and then with DMEM (4).