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Control of the Normal and Pathological Development of Neural Stem and Progenitor Cells by the PC3/Tis21/Btg2 and Btg1 Genes

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The PC3/Tis21/Btg2 and Btg1 genes are transcriptional cofactors belonging to the Btg/Tob family, which regulate the development of several cell types, including neural precursors. We summarize here the actions of these genes on neural precursors in the adult neurogenic niches and the cognitive defects associated when their expression is altered. We consider also recent findings implicating them in neural and non-neural tumors, since common developmental mechanisms are involved. PC3/Tis21 is required for the regulation of the maturation of stem and progenitor cells in the adult dentate gyrus and subventricular zone (SVZ), by controlling both their exit from the cell cycle and the ensuing terminal differentiation. Such actions are effected by regulating the expression of several genes, including cyclin D1, BMP4, Id3. In cerebellar precursors, however, PC3/Tis21 regulates chiefly their migration rather than proliferation or differentiation, with important implications for the onset of medulloblastoma, the cerebellar tumor. In fact PC3/Tis21 is a medulloblastoma-suppressor, as its overexpression in cerebellar precursors inhibits this tumor; PC3/Tis21 shows anti-tumor activity also in non-neural tumors. Btg1 presents a different functional profile, as it controls proliferation in adult stem/progenitor cells of dentate gyrus and SVZ, where is required to maintain their self-renewal and quiescence, but is apparently devoid of a direct control of their terminal differentiation or migration. Notably, physical exercise in Btg1-null mice rescues the loss of proliferative capability occurring in older stem cells. Both genes could be further investigated as therapeutical targets, namely, Btg1 in the process of aging and PC3/Tis21 as a tumor-suppressor.

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The Btg2 (or PC3 in rat and Tis21 in mouse) and Btg1 genes are transcriptional cofactors belonging to the Btg/Tob family, that regulate division and differentiation of several cell types. This review aims to describe the functional roles of Btg2 and Btg1 in the generation of new neurons in the adult brain, in relation to the control of the cell cycle and differentiation of neural stem cells, and to the development of cerebellar tumors as a consequence of a misregulation of the process of neurogenesis in the cerebellum. A summary of the most recent findings on the implication of the Btg genes in other neural and non-neural tumors is also provided, as common mechanisms of action are present.

PC3/Tis21, regulator of transcription and cell cycle

PC3 was initially isolated in a neural crest-derived line, the rat pheochromocytoma PC12 cells, at the initial steps of the differentiation into sympathetic neurons induced by nerve growth factor (Bradbury et al., 1991). Concomitantly, the mouse orthologue Tis21 was identified as a phorbol ester-induced gene in NIH3T3 mouse fibroblasts (Fletcher et al., 1991); Btg2 is the human orthologue (Rouault et al., 1996).

PC3/Tis21 (as we refer here to PC3/Tis21/Btg2) regulates transcription by associating with the promoters of several genes, including cyclin D1 (Farioli-Vecchioli et al., 2007), RARβ (Passeri et al., 2006), Id3 (Farioli-Vecchioli et al., 2009) and Cxcl3 (Farioli-Vecchioli et al., 2012a), and acting as a component of protein complexes. These can contain histone modifying factors to which PC3/Tis21 is known to bind, such as the methyltransferase Prmt1 (Lin et al., 1996) and the histone deacetylases HDAC4 or HDAC1 (Passeri et al., 2006; Farioli-Vecchioli et al., 2007), and/or transcriptional elements such as Caf1/CNOT8 (Rouault et al., 1998; Prévôt et al., 2001), or the transcription factor HoxB9 (Prévôt et al., 2000).

Today, more than one hundred reports link PC3/Tis21 to the cell cycle as a negative regulator, in neural and non-neural cells as well as in tumor cells. For example, PC3/Tis21 was found able to arrest the cell cycle progression in PC12 cells (Montagnoli et al., 1996; el-Ghissassi et al., 2002); furthermore, PC3/Tis21 synergizes with nerve growth factor in inducing their differentiation (Corrente et al., 2002; el-Ghissassi et al., 2002). Similarly, overexpression of PC3/Tis21 induces arrest in the G0-G1 phase of the cell cycle in non-neural cells, such as normal fibroblasts (NIH3T3 cells; Guardavaccaro et al., 2000), mouse embryo fibroblasts and embryonic stem cells (Rouault et al., 1996; Boiko et al., 2006), granulosa cells of the ovary (Li et al., 2009), or breast (Kawakubo et al., 2004) and prostate cancer cells (Ficazzola et al., 2001). Such G0-G1 phase arrest is consequent to inhibition of the expression of cyclin D1 and of the activity of Cdk4/cyclin D1 complexes on pRb

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(Guardavaccaro et al., 2000; Kawakubo et al., 2004; Boiko et al., 2006). Interestingly, a pRb-independent mechanism of inhibition of the G1/S phase by PC3/Tis21 has been found to involve the reduction of cyclin E synthesis and cdk4 activity, in human embryonic kidney 293 cells (Lim et al., 1998).

Moreover, PC3/Tis21 has been involved also in the inhibition of the G2/M phase of the cell cycle, mainly in DNA-damaged mouse embryonic stem cells (Rouault et al., 1996), in monocyte cells (Kim et al., 2014) or in tumor cells. In this latter regard, the up-regulation of PC3/Tis21 correlates with G2/M arrest in U937 monocytic tumor cells, by inhibiting cyclin B1-Cdc2 binding (Ryu et al., 2004); similarly, PC3/Tis21 inhibits the proliferation of transformed hepatocytes by disrupting cyclin B1-cdk1 activity (Park et al., 2008).

Role of PC3/Tis21 in the control of stem/progenitor cells in the adult dentate gyrus and subventricular zone

New neurons are constantly generated in the adult brain within two neurogenic niches, the dentate gyrus of the hippocampus and the subventricular zone (SVZ), adjacent to the lateral ventricle (Bayer et al., 1982; Cameron et al., 1993; Alvarez-Buylla and Lim, 2004). These new neurons have been shown to be required for hippocampus-dependent learning and memory and for olfactory memory, respectively (Zhao et al., 2008a; Lazarini and Lledo, 2011).

In the dentate gyrus the new neurons arise from stem cells with radial glial-like morphology (Seri et al., 2001; Graham et al., 2003; Komitova and Eriksson, 2004; Kempermann et al., 2004) that mature in two stages of progenitor cells (named type-2ab and type-3), to finally become early postmitotic and terminally differentiated neurons (stage 5 and stage 6, respectively; Filippov et al., 2003; Fukuda et al., 2003; Kronenberg et al., 2003; Brandt et al., 2003). Similarly, the adult neurons of the SVZ derive from a subset of astrocytes corresponding to quiescent neural stem cells (B cells), which evolve into transit amplifying cells and neuroblasts (C and A cells, respectively) that migrate to the olfactory bulb (Doetsch et al., 2002; Alvarez-Buylla and Lim, 2004; Zhao et al., 2008a).

PC3/Tis21 is physiologically expressed in neural progenitor cells of different areas of the adult murine brain, such as the cerebellum, the hippocampus and the SVZ, and induces them to exit the proliferative state and differentiate (Canzoniere et al., 2004; Farioli-Vecchioli et al., 2007, 2008, 2009; Attardo et al., 2010). Evidence obtained with short pulses of BrdU incorporation indicates that PC3/Tis21 is endowed with an intrinsic inhibitory action of the S-phase in progenitor cells of the dentate gyrus (Farioli-Vecchioli et al., 2009). Indeed, overexpression of PC3/Tis21 in dentate gyrus progenitor cells, in a transgenic mouse model, decreases the number of cells incorporating BrdU (Farioli-Vecchioli et al., 2008), while its ablation increases this number and appears to reduce the length of the G1 phase of dentate gyrus progenitor cells (Farioli-Vecchioli et al., 2009). In fact, the ratio of progenitor cells in S-phase ($BrdU^+$) to those actively dividing ($Ki67^+$) increases in PC3/Tis21 knockout progenitor cells, this ratio being inversely proportional to the length of the cell cycle and/or of the G1-S phase (Farioli-Vecchioli et al., 2009). The inhibition of the cell cycle by PC3/Tis21 is likely to result in an increase of the asymmetric neurogenic division of progenitor cells, which might well explain the accelerated differentiation of dentate gyrus progenitor cells occurring without change in the final number of neurons generated, after overexpression of PC3/Tis21 (Farioli-Vecchioli et al., 2008). This would also be consistent with the findings that: i) during brain development expression of PC3/Tis21 is associated to proliferating neuroblasts undergoing a neurogenic division (Iacopetti et al., 1994, 1999); ii) telencephalic neural progenitors switching from proliferative to neuron-generating division express PC3/Tis21 and present a lengthening of the cell cycle, as measured by BrdU cumulative labeling, suggesting this as a general mechanism to start the neuron-generating division (Calegari et al., 2005).

However, PC3/Tis21 has also a pro-differentiative action intrinsic and independent of the antiproliferative action. This was suggested by the group of Gerd Kempermann, observing that in a Tis21 GFP-knockin mouse Tis21 was expressed not only in proliferating dentate gyrus cells but also selectively in postmitotic neurons (stage 5; Attardo et al., 2010), and was demonstrated by the observation that stage 5 early postmitotic dentate gyrus neurons lacking PC3/Tis21 are unable to terminally differentiate into stage 6, although they have already exited the cell cycle (Farioli-Vecchioli et al., 2009). Thus, PC3/Tis21 acts according to a two-step process, i.e., arrest of the cell cycle, followed by terminal differentiation. This is also evident in the stem cells of the adult SVZ, where ablation of PC3/Tis21 leads to increase of their proliferation and impairment of the terminal differentiation of neuroblasts (A cells) (Farioli-Vecchioli et al., 2014a).

Notably, the pro-differentiative action of PC3/Tis21 occurs only in already postmitotic cells; thus, in order to differentiate it is first necessary that the neural stem cell exits the cycle, becoming postmitotic (Farioli-Vecchioli et al., 2009; Attardo et al., 2010). This appears to be a general mechanism throughout neural differentiation, which first requires an inhibitor of the cell cycle, as pointed out by Calegari et al. (2005) such as PC3/Tis21 itself or another gene induced by PC3/Tis21. It is worth noting that a stimulus inducing the proliferation of dentate gyrus progenitor cells, such as the overexpression of cyclin D1, is unable to increase the number of differentiated neurons unless is preceded by a period of arrest of the proliferation to allow differentiation to occur, consistently with the need of a two-step process (Artegiani et al., 2011).

On the whole, the functional profile of PC3/Tis21 appears that of a pan-neural activator of the transition from neural progenitor cell to early postmitotic and then to terminally differentiated neuron (see Fig. 1A). Table 1 summarizes the altered phenotypes observed in different neural areas of PC3/Tis21 transgenic and mutant mouse models (including the cochlear neuroepithelium and neocortex, see below).

Notably, an alteration of the physiological expression of PC3/Tis21 in neural progenitor cells causes cognitive impairments dependent on the dentate gyrus and SVZ. In fact, overexpression of PC3/Tis21 in dentate gyrus progenitor cells, which induces an acceleration of differentiation, results in a very strong deficit of the hippocampus-dependent memory, i.e., of the spatial and associative memory (Farioli-Vecchioli et al., 2008). If, on the contrary, PC3/Tis21 is ablated, the ensuing impairment of terminal differentiation of dentate gyrus neurons is associated to a selective loss of associative memory (Farioli-Vecchioli et al., 2009), while the impaired differentiation of the SVZ/olfactory bulb neurons is associated to a loss of olfactory discrimination (Farioli-Vecchioli et al., 2014a). Clearly, these findings point to to a general paradigm, i.e., how the timing of differentiation of a neuron is extremely critical for its function (for discussion, Tirone et al., 2013).

Neural pathways involving PC3/Tis21 function

In the absence of PC3/Tis21, progenitor cells of the dentate gyrus and stem cells of the SVZ show a great increase of the Id3 protein - which is an inhibitor of proneural basic helix-loop-helix (bHLH) genes (Lyden et al., 1999; Yokota, 2001) - and also, at least in SVZ, an increase of cyclins D, A and E and a very strong decrease of BMP4 expression (Farioli-Vecchioli et al., 2009, 2014a). BMP4 is known to maintain the quiescence of stem cells in the dentate gyrus and

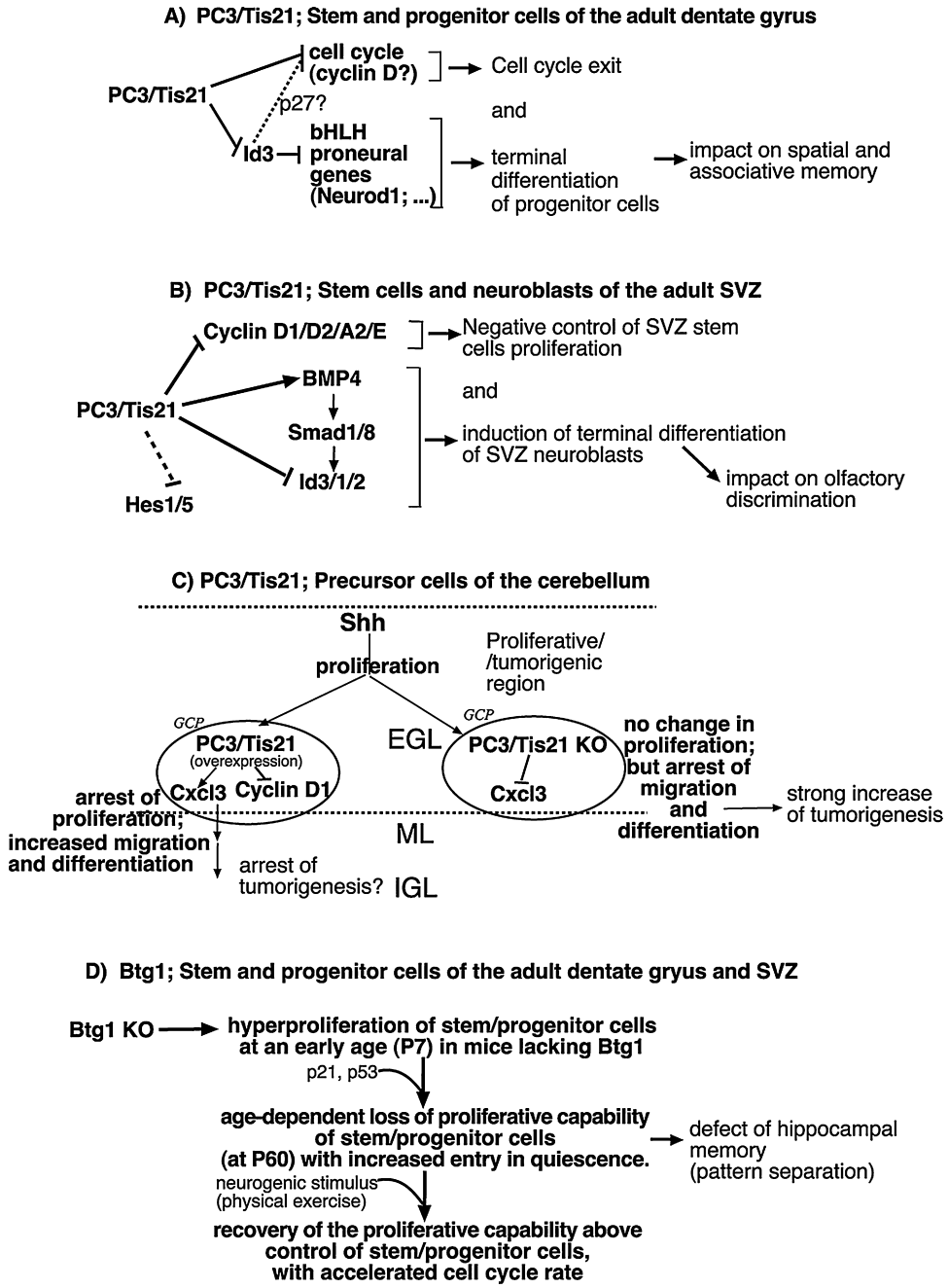

Fig. 1. Working model for the action of PC3/Tis21 in the (A) adult dentate gyrus, (B) adult subventricular zone and (C) postnatal cerebellum, and (D) of Btg1 in dentate gyrus and SVZ. (A) Dentate gyrus. In the stem/progenitor cells of the dentate gyrus, PC3/Tis21 induces differentiation by directly inhibiting Id3 (Farioli-Vecchioli et al., 2009), inhibitor of proneural genes. Furthermore, as Id3 may inhibit p27 expression (Kee and Bronner-Fraser, 2005), PC3/Tis21 may inhibit the cell cycle also through the negative regulation of Id3 (see text). It has yet to be defined whether PC3/Tis21 inhibits the cell cycle in the dentate gyrus also through cyclin D1 or, rather, cyclin D2 (considered, in the hippocampus, more critical than cyclin D1: Kowalczyk et al., 2004). Scheme modified from Farioli-Vecchioli et al. (2009). (B) SVZ. The ablation of PC3/Tis21 in the SVZ elicits an increase of proliferation of the stem cells and the expression of several cyclins as well as of Id3, whereas pro-differentiative genes such as NeuroD1 and BMP4 are downregulated, consistently with the idea that PC3/Tis21 is required for cell cycle arrest and differentiation. Remarkably, the arrest of differentiation observed in primary SVZ cells is reverted by BMP4 and by Id3 silencing. Modified from Farioli-Vecchioli et al., (2014a). (C) Postnatal cerebellum. At the cerebellar surface (EGL) Sonic hedgehog (Shh) triggers the proliferation of the granule neuron precursor cells (GCPs). Overexpression of PC3/Tis21 inhibits the proliferation of the GCP (through cyclin D1; Canzoniere et al., 2004) and triggers their differentiation and migration (Farioli-Vecchioli et al., 2012a), thus reducing tumor frequency in Patched1 mice (Farioli-Vecchioli et al., 2007). When PC3/Tis21 is silenced no effect on the proliferation of GCPs is observed, but their migration is impaired. ML, molecular layer; IGL, internal granular layer. (D) The ablation of Btg1 triggers hyperproliferation in stem/progenitor cells of the dentate gyrus and SVZ of young mice, followed by an age-dependent decrease of the proliferation and by quiescence. A neurogenic stimulus such as physical exercise reactivates the proliferation of Btg1-null stem cells, suggesting that they hold a potential for rejuvenation.

TABLE 1. Neural phenotypes observed in PC3/Tis21 transgenic and knockout mouse models.

Gain or loss-of-function	Cell type genetically modified	Effect (morphology/phenotype)	Cause (cellular and molecular parameters altered)	Ref
Neural tube at E12.5 (cervical level)				
Tis21 overexpression	Expressed in GCPs since conception (nestin driven)	Greater number of new neurons generated in the neural tube	Inhibition of cell cycle (marked decrease of BrdU incorporation in the VZ) Increased generation of new neurons (2-fold increment of $Tis21^{+}$/βIII $tubulin^{+}$ and MAP-2-positive cells; no increase of apoptosis)	Canzoniere et al., 2004
Adult dentate gyrus stem and progenitor cells (P60)				
Tis21 overexpression	Expressed in stem/progenitor cells from P30 until P95 (nestin driven)	No significant morphological change in hippocampus or dentate gyrus volume	Inhibition of cell cycle in stem and progenitor cells (type-1-3), but no change in total BrdU incorporation (compensatory increase of $BrdU^{+}$ early differentiated neurons). Accelerated differentiation of stage 5 and stage 6 new neurons 1-5- and 28-day-old; no change in final number of neurons. Possible increase of asymmetric divisions. Reduced LTP of dentate gyrus neurons; reduced episodic and associative memory.	Farioli-Vecchioli et al., 2008
Tis21 knockout (constitutive)	Stem/progenitor cells	No significant morphological change in hippocampus or dentate gyrus volume; but specific impairment of the terminal differention of dentate gyrus neurons	Acceleration of cell cycle (increased number of dentate gyrus progenitor cells entering in S-phase [$BrdU^{+}$]; reduced length of G1 phase in progenitor cells [$BrdU^{+}$/$Ki67^{+}$]) Impaired terminal differentiation (increase of stage 5 neurons [$NeuN^{+}Calretinin^{+}$] and decrease of stage 6 neurons [$NeuN^{+}Calbindin^{+}$]). Increase of expression of the antineural gene Id3 (loss of the inhibition exerted from Tis21 by binding the promoter of Id3) Reduced associative memory.	Farioli-Vecchioli et al., 2009
Tis21 GFP-knockin (constitutive)	Stem/progenitor cells	N.D.	Tis21-expression (as detected by GFP) is bimodal: firstly is associated with type-2 progenitor cells, then to stage 6 terminally differentiated neurons. Hypothesis for an additional specific role of Tis21 in terminal differentiation.	Attardo et al., 2010
Adult subventricular zone stem cells and neurons (P60)				
Tis21 knockout (constitutive)	B Stem cells/C transient amplifying cells/A neuroblasts	Reduced number of differentiated SVZ-derived neurons in olfactory bulb.	Maintenance of stem cells quiescence, as Tis21-null proliferating $BrdU^{+}GFAP^{+}$ B stem cells increase; consistently D, A and E cyclins increase. Impaired terminal differentiation of A neuroblasts (DCX^{+}) in Tis21-null SVZ; a rescue experiment with BMP4 and with an shRNA targeting Id3 indicates that the impairment of terminal differentiation of A cells depends on the increase of Id3 and decrease of BMP4. Reduced number of neurons in the olfactory bulb as a consequence of reduced number of terminally differentiated neurons in SVZ: increased olfactory thershold.	Farioli-Vecchioli et al., 2014
Cerebellum – granule neuron precursor cells				
Tis21 overexpression	Expressed in GCPs since conception until P5 (nestin driven)	Decrease of cerebellar size	Inhibition of cell cycle (decrease of BrdU incorporation, reduced cyclin D1expression) Increased differentiation (induction of Neurofilament 160k and Math1 expression)	Canzoniere et al., 2004
Tis21 overexpression	Expressed in GCPs since conception until adulthood (β-actin driven)	Large decrease of cerebellar size (55% decrease, in 6–8% of mice)	Inhibition of cell cycle (decrease of BrdU incorporation, reduced expression of cyclin D1) Increased differentiation (induction of Neurofilament 160k and Math1 expression)	Canzoniere et al., 2004
Tis21 overexpression	Expressed in GCPs since the perinatal stage (β-actin driven)	N.D.	Inhibition of cell cycle (decrease of BrdU incorporation, reduced expression of cyclin D1; no change in cyclin D2 or cyclin B1 expression) Increased differentiation ($NeuroD1^{+}$ neurons)	Farioli-Vecchioli et al., 2007

Table 1. (Continued)

Gain or loss-of-function	Cell type genetically modified	Effect (morphology/phenotype)	Cause (cellular and molecular parameters altered)	Ref
Tis21 knockout (constitutive)	GCPs, from embryogenesis	N.D.	No effect on cell cycle (unchanged BrdU incorporation in GCPs) Small but significant decrease of differentiation (NeuroD1 and NeuN) Large decrease of migration of GCPs out of the surface of cerebellum to the internal layers (inhibition of Cxcl3 expression)	Farioli-Vecchioli et al., 2012a
Neocortex				
Tis21 overexpression	Expressed in neural stem cells since conception (nestin driven)	Decrease of cortex size (30% decrease)	N.D.	Canzoniere et al., 2004
Tis21 overexpression	Expressed by electroporation in dorsolateral telencephalon of E13.5 embryo	Selective decrease of cortex size	No apparent change of the total BrdU incorporation in neocortex progenitor cells. Decrease of neurogenesis selective for cortex neurons; increase of symmetric neurogenic divisions.	Fei et al., 2014
Cochlea				
Tis21-GFP knock-in (constitutive)	Expressed in spiral ganglion cells of the cochlea since E13.5	The development of Rosenthal's canals was inhibited in Tis21-GFP knock-in mice.	The number of spiral ganglion cells decreased in Tis21-GFP knock-in mice. Possible cause is an impairment in the shift from proliferation to differentiation and maturation of cochlear ganglion neurons.	Yamada et al., 2014

N.D. = not determined.

SVZ (Lim et al., 2000; Mira et al., 2010). Thus, PC3/Tis21 inhibits the expression of cyclins and Id3 and induces that of BMP4. Notably, the silencing of Id3 or the treatment with recombinant BMP4 protein fully rescue the defect of terminal differentiation in PC3/Tis21-null SVZ neurosphere cells, indicating that Id3 and BMP4 are both responsible for the PC3/Tis21-dependent impairment of terminal differentiation of the neurons (Farioli-Vecchioli et al., 2014a). As Id3 and BMP4 appear to be anti- and pro-differentiative, respectively, a fair possibility is that PC3/Tis21 acts as upstream controller of the opposite actions they exert in the SVZ (see Fig. 1B**)**. Furthermore, the fact that in the PC3/Tis21 null dentate gyrus the number of Id3-positive proliferating progenitor cells increases significantly (Farioli-Vecchioli et al., 2009) suggests that PC3/Tis21 may inhibit the cell cycle also through a negative regulation of Id3 (which may in turn inhibit p27: Kee and Bronner-Fraser, 2005; Fig. 1A**)**.

Role of PC3/Tis21 in the control of cerebellar progenitor cells and tumorigenesis

PC3/Tis21 is expressed physiologically in the precursors of cerebellar granule neurons (GCPs) as early as they are generated from the rhombic lip (Canzoniere et al., 2004), a germinative epithelium at the roof plate of the fourth ventricle, from which GCPs migrate at the surface of the prospective cerebellum to form the external granular layer (EGL) (Hatten, 1999; Wang and Zoghbi, 2001). The cerebellum develops postnatally from the EGL, through migration of the GCPs to the internal layers of the cerebellum where they differentiate (Rakic, 1971). Early after birth the GCPs undergo an intensive proliferation in the EGL under the proliferative stimulus of Sonic Hedgehog (Shh; Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Importantly, the prolonged mitotic activity to which the GCPs are subject at the surface of the cerebellum during the postnatal morphogenesis, makes them potential targets of transforming insults (Wang and Zoghbi, 2001). This can lead to the formation of medulloblastoma (MB), which, in at least 25% of cases, originates from GCPs presenting a misregulation of their proliferative pathways (Yang et al., 2008; Gibson et al., 2010). Today four types of MB have been identified (Wnt-associated, Shh-associated, group 3 and 4), which develop from different cellular origins (see Box 1); MB is clearly of multigenic origin and the expression of PC3/Tis21 has been shown to be reduced in several MBs, mainly those Shh-dependent (Farioli-Vecchioli et al., 2007). The overexpression of transgenic PC3/Tis21 in GCPs exerts a powerful inhibitory action on the proliferation of normal and neoplastic GCPs, favoring their differentiation and reducing the frequency of tumors in a Shh-activated mouse model of spontaneous MB (Patched1$^{+/-}$; Farioli-Vecchioli et al., 2007). This indicates that PC3/Tis21 is a MB suppressor; consistently, the frequency of spontaneous MB in the Patched1$^{+/-}$ mouse highly increases if PC3/Tis21 is ablated (Patched1$^{+/-}$/Tis21KO mice; Farioli-Vecchioli et al., 2012a; see Table 1).

Interestingly, overexpression of PC3/Tis21 in GCPs inhibits their proliferation through a mechanism involving the repression of cyclin D1 and recruitment of PC3/Tis21 to the cyclin D1 promoter accompanied by histone deacetylation (Farioli-Vecchioli et al., 2007); in contrast, ablation of PC3/Tis21 in the cerebellum causes a significant impairment of migration and differentiation of GCPs, but does not affect their proliferation (Farioli-Vecchioli et al., 2012a). Preliminary data indicate that the antiproliferative action of PC3/Tis21 in the cerebellum is vicariated by the family-related gene Btg1 (M. Ceccarelli and L. Micheli, unpublished data; Farioli-Vecchioli et al., 2012b); this further suggests that the

Box 1 Different embryonic origin of MB

Four molecular subtypes of MBs have been identified, molecularly different and with distinct cellular origins (Shh-type, Wnt-type, groups 3 and 4).

Three different progenitor cell populations within the rhombic lip - a germinative epithelium at the roof plate of the fourth ventricle - have been identified as possible origins for MB. First, cerebellar granule neuron precursors (GCPs) derived from the upper rhombic lip (URL) give rise to Shh-type MBs (Schüller et al., 2008); second, cochlear granule neuron precursors from the auditory rhombic lip generate Shh-type MBs that develop near to the cochlear nuclei (Grammel et al., 2012); and, third, pontine grey neuronal progenitors generated from the lower rhombic lip have been suggested as a source for Wnt-type MBs (Gibson et al., 2010). These findings were based on the different expression of specific genes in GCPs: i) in Shh-type MBs the genes of the Shh pathway as well as Math1 (which is responsible for the specification and differentiation of GCPs) had altered expression; ii) mice mutated for the Wnt pathway effector CTNNB1 showed altered expression of Zic1 and defects of migration of neurons from lower rhombic lip to the the dorsal and ventral brainstem (no change being observed for proliferation or apoptosis). The latter altered genetic and cellular pattern was associated to Wnt-type MBs developing outside the cerebellum, in the dorsal brainstem (Gibson et al., 2010). Interestingly, also group 3 MBs have been proposed to derive from GCPs (Northcott et al., 2012), while the cellular origin of MB group 4 is unknown. As the expression of Tis21 matches that of Math1 (Canzoniere et al., 2004), it is plausible to think that Tis21 is involved in Shh-dependent MBs (see text).

antiproliferative and pro-differentiative actions of PC3/Tis21 are dissociable.

Moreover, we and others (Haag et al., 2012; Farioli-Vecchioli et al., 2012a) have pointed out how the timing of migration of preneoplastic GCPs (pGCPs) during cerebellar development is critical for their malignant progression. These reports show that ablation of PC3/Tis21 or of Nos2 (nitric oxide synthase) hinders the migration of both normal and neoplastic GCPs from the surface of the cerebellum toward the internal layers. This is a consequence of the decrease of expression of Gap43 or of the chemokine Cxcl3, which specifically induce GCPs migration and are activated by Nos2 and PC3/Tis21, respectively. Ablation of either Nos2 or PC3/Tis21 in Shh-activated mice causes a large increase in the frequency of MB. Plausibly, a prolonged permanence in the external proliferative cerebellar region under control of Shh highly increases the probability of neoplastic transformation. A relevant observation is that treatment with Cxcl3 ex vivo of cerebellar slices from Shh-activated mice causes a decrease of the area of tumor lesions (Farioli-Vecchioli et al., 2012a). The underlying mechanism is that neoplastic GCPs can still differentiate and migrate like normal GCPs, although they are able to generate a tumor when transplanted (Kessler et al., 2009). This implies that neoplastic cerebellar precursors can be forced to differentiate and exit the neoplastic program, by inducing them to migrate from the Shh-controlled proliferative region at the surface of the cerebellum through the migration-promoting action of Cxcl3 or Gap43. Thus, these studies raise the possibility of controlling the development of MB by regulating the migration of GCPs. This would offer the possibility of a novel therapy of MB. Certainly, there are caveats to be evaluated; for instance, after a yet undefined period of time, pGCPs may become irreversibly transformed and lose the ability to differentiate. If so, it is necessary that the treatment with proteins regulating GCP migration such as Cxcl3 or Gap43 takes place at the very initial stages of the tumor. See summary in Figure 1C.

Evidence for co-regulation of GCP development by PC3/Tis21 and Math1

A further issue concerns the observation that PC3/Tis21 appears to regulate Math1 expression in GCPs. In fact, the profiles of expression of PC3/Tis21 and Math1 in GCPs overlap since early embryonic stages in the rhombic lip (Canzoniere et al., 2004). Furthermore, the overexpression of PC3/Tis21 in GCPs causes an induced expression of Math1 in GCPs, whereas the ablation of PC3/Tis21 causes a decrease (Canzoniere et al., 2004; Farioli-Vecchioli et al., 2013). This is relevant considering that Math1 specifies the fate of GCPs: in its absence GCPs are generated from the rhombic lip, but do not differentiate and the EGL is never formed (Ben-Arie et al., 1997; Gazit et al., 2004). Moreover, if Math1 is silenced, no MB cell develops (Zhao et al., 2008b; Flora et al., 2009). An interesting possibility was proposed by Flora et al. (2009). When cerebellar precursors are in a Shh-dependent proliferative environment, Math1 makes the cells competent to transduce the proliferative signal of Shh and promotes the MB. In contrast, when the cells are exposed to differentiative signals, Math1 has a pro-differentiative action, that prevents the MB. We have previously proposed that PC3/Tis21 may induce the differentiation of GCPs by inducing Math1 (Canzoniere et al., 2004), and in keeping with this idea, it is possible that the ablation of the pro-differentiative gene PC3/Tis21 in Shh-activated mice, depriving the GCPs of a differentiative stimulus, could favor the pro-Shh action of Math1. Consequently, the action of activated Shh on GCPs at the surface of the cerebellum would became more penetrant.

Reduction of cerebellum and neocortex volume (microcephaly) by PC3/Tis21 overexpression during embryogenesis

An interesting phenotype associated to continued overexpression of PC3/Tis21 in GCPs and in cortical precursors since conception consists in a decrease of the size of cerebellum (up to 55%) and of the neocortex, in 6–8% of mice (Canzoniere et al., 2004). Similarly, cyclin D1/D2-null mice show a selective decrease of the cerebellar size (Ciemerych et al., 2002), also suggesting that at least in part D-type cyclins may be involved in the PC3/Tis21-dependent phenotype, as cyclin D1 is clearly down-regulated in GCPs overexpressing PC3/Tis21 (while cyclin D2 is barely affected) (Canzoniere et al., 2004). Moreover, recently Fei et al. (2014) observed that overexpressing Tis21 (mouse sequence) in embryonic brain after E13.5 (by removing an inhibitory region at the 3′UTR of Tis21, targeted by miRNA-92) led to a selective impairment in the growth of the neocortex, with a phenotype similar to microcephaly, a developmental pathology. Fei et al. conclude that the expansion of neocortex precursors is inhibited by Tis21 without acting on the cell cycle but by altering the mode of cell division; this idea comes, however, from an experiment of cumulative BrdU labeling of the total population of neural cells of the ventricular zone at E14.5, without distinction between subpopulations. As the authors perform electroporation of the Tis21 mutated construct in the dorsolateral telencephalon of E13.5 embryos, it would be interesting to see whether an electroporation at a later developmental stage could affect also the cerebellum, as at E13.5 a limited number of cerebellar precursors exist in the rhombic lip.

Implication of Btg2 in gliomas and in non-neural tumors

The Btg2 gene (as we refer to PC3/Tis21/Btg2 throughout this section) is generally considered an antioncogenic gene (Matsuda et al., 2001; Tirone, 2001; Duriez et al., 2004; Lim, 2006). The loss of Btg2 has been implicated in cell transformation in several different tumors, including another neural tumor such as the glioma (Appolloni et al., 2012) or non-neural such as prostate (Ficazzola et al., 2001 Coppola et al., 2013), lung (Wei et al., 2012), breast (Kawakubo et al., 2006; Takahashi et al., 2011), bladder (Wagener et al., 2013), and liver tumors (Park et al., 2008). Low or absent levels of Btg2 expression in tumors have been shown to generally correlate with a less favourable clinical prognosis (Möllerström et al., 2010; Jalava et al., 2012). Notably, point mutations in the Btg2 gene have been reported in B cell malignancies (Morin et al., 2011).

A very evident role of Btg2 in neural tumorigenesis has been recently pinpointed by reports showing that in a mouse model of PDGF-induced gliomas Btg2 plays a critical role in tumor progression and that patients with gliomas presenting low levels of Btg2 expression have a significantly worse prognosis (Calzolari et al., 2008; Appolloni et al., 2012).

Other recent reports demonstrated an important role for Btg2 in cellular migration during tumorigenesis. In particular, it was shown that the endogenous expression of Btg2 contributes to the migratory potential of bladder cancer cells and that high levels of Btg2 in these cells are linked to decreased cancer-specific survival (Wagener et al., 2013). However, such positive correlation between Btg2 expression and migration capacity contrasts with the observations in breast cancer cells where these parameters exhibited an inverse correlation (Takahashi et al., 2011); similarly, another study revealed that Btg2 negatively regulated cancer cell migration by inhibiting Src activity through downregulation of ROS generation in mitochondria (Lim et al., 2012). Altogether, keeping in account also our studies on the migration of cerebellar precursors, it seems evident that Btg2 can exert differential effects on the cellular migration depending on the cellular context, and that this action plays a role in tumorigenesis.

Btg2 is involved in different pathways of tumor cells, as Btg2 is a target of p53 and induces suppression of Ras-induced transformation (Boiko et al., 2006). More recently, Lim and colleagues demonstrated that Btg2 activates Erk1/2 and inhibits Akt in response to all-trans-retinoic acid during differentiation of acute promyelocytic leukemia HL-60 cells, in this way cooperating to down-regulate c-myc and exerting an anticarcinogenic potential (Imran et al., 2012).

A further way by which PC3/Tis21 may exert its antioncogenic activity is also by accelerating DNA repair through Prmt1 methylation, which prevents damage signals from Chk2(T68)-p53(S20) (Choi et al., 2012).

Btg1 is required to maintain the pool of stem cells in the adult dentate gyrus and subventricular zone

Among the genes of the Btg/Tob family, Btg1 is the one more closely related to PC3/Tis21 (65% homology; Rouault et al., 1992; Tirone, 2001). Also Btg1 is required for the control of the proliferation of stem/progenitor cells in the dentate gyrus and SVZ, as cycling progenitor cells ablated of Btg1 increase strongly in number (labeled as $Ki67^+$ or $BrdU^+$ cells after a short BrdU pulse), as observed for PC3/Tis21 (Farioli-Vecchioli et al., 2012b). Such an increase, however, is transient, being evident only during the early postnatal period (with a peak at P7), whereas in adult Btg1-null mice (2-month-old) the total pool of proliferating dentate gyrus and SVZ cells is reduced, relative to control mice. Consistently, the Btg1-null progenitor cells at P7 show a strong preference to be in cycle, as judged from the reduced number of $BrdU^+Ki67^-$ cells that have entered the S-phase and then ceased to cycle, whereas the adult Btg1-null stem/progenitor cells exit from the cell cycle and become quiescent with much higher frequency than wild-type. Such an exit is concomitant with a several-fold increase, relative to the wild-type, of stem cells (type-1) expressing p21 or p53 and is followed within a few days by apoptosis (Farioli-Vecchioli et al., 2012b).

Thus, the ablation of Btg1 results primarily in a disinhibition of the cell cycle, accompanied by apoptosis - a normal event after suppression of a negative regulator of the cell cycle (Lee et al., 1994) – and is secondarily followed by an age-dependent decrease of the proliferative capacity of progenitor cells. Accordingly, the length of the S phase increases considerably in Btg1-null adult dentate gyrus stem cells and progenitor cells, as recently observed by a novel technique for cell cycle kinetics analysis (Farioli-Vecchioli et al., 2014b). Hence, it appears that at the origin of the age-dependent decrease of the proliferative capability of Btg1 knockout stem cells is a compensatory increase of the cell cycle inhibitors p21 and p53, which drives the cells to quiescence. This would explain why the pool of stem cells is evidently not fully depleted in the Btg1-null adult dentate gyrus, but can recover its proliferative capability if stimulated (see below).

Notably, primary SVZ neurospheres from adult Btg1-null mice show a strong reduction of the ability to replicate by asymmetric division, responsible for self-renewal, whereas the opposite occurs in primary neurospheres from P7 Btg1-null mice (Farioli-Vecchioli et al., 2012b). Thus, the lengthening of the cell cycle appears to be not necessarily associated to the induction of asymmetric division (see above), which suggests that the correlation between inhibition of cell cycle and asymmetric division in neural cells, although rich of circumstancial evidence (see Malumbres, 2011 for review), is likely to involve other regulatory cues controlling the division mode of progenitor cells.

As a whole, the reduced number of adult neurons generated in Btg1-null dentate gyrus and olfactory bulb (where the neurons generated in SVZ migrate) is basically the consequence of both apoptosis and loss of proliferative ability (quiescence) of the pool of stem and progenitor cells, rather than of an impairment of differentiation. Altogether, Btg1 appears to be necessary for the maintenance and self-renewal of stem cells in the adult dentate gyrus and SVZ; interestingly, the phenotype of loss of the stem cell pool, observed after removal Btg1, is present also in the knockout of p21 (Kippin et al., 2005) or of the Notch effector RBPJ (Imayoshi et al., 2010).

Thus, the functional profile of Btg1 does not fully overlap that of PC3/Tis21, which seems to play a crucial role in terminal differentiation of the adult dentate gyrus and SVZ neurons (Farioli-Vecchioli et al., 2009, 2014a; Attardo et al., 2010). Moreover, Btg1 is not induced by p53 (Cortes et al., 2000), unlike PC3/Tis21, which acts in a pathway parallel to p21, being induced by p53 as is p21 (Rouault et al., 1996; Sionov et al., 2000).

Functionally, the reduced number of adult neurons in the dentate gyrus of Btg1-null mice is associated to fine impairments in hippocampus-dependent learning and memory, i.e., in pattern separation, which is the ability to discriminate among potentially overlapping experiences of episodic memory. This is also consistent with the notion that adult neurogenesis is expected to enhance the extent of information encoded by the dentate gyrus (Farioli-Vecchioli et al., 2012b; Fig. 1D).

Implication of Btg1 in non-neural tumors

So far, neural tumors involving Btg1 have not been identified. However, the level of expression of Btg1 is directly

proportional to the inhibition of proliferation and tumor metastasis, and to the induction of apoptosis in a variety of non-neural tumor cells; namely, in breast (Zhu et al., 2013; Li et al., 2014), ovarian (Zhao et al., 2013), thyroid (Lu et al., 2014), liver (Sun et al., 2014a), nasopharyngeal (Sun et al., 2014b), and non-small cell lung cancers (Sun et al., 2014c). Moreover, deregulation of Btg1 by gene deletions, point mutations or chromosomal translocations is frequently observed in B cell malignancies (Rimokh et al., 1991; Lohr et al., 2012; Waanders et al., 2012). Remarkably, functional parallels are present in the role played by Btg1 and PC3/Tis21 in the maturation of blood and neural precursor cells (B. Scheijen, personal communication). Furthermore, given that the Btg1 mRNA is expressed in the cerebellum (Farioli-Vecchioli et al., 2012b, 2014b), an involvement of the gene also in the development of MB is possible.

neurogenic

Recovery of the Btg1-dependent loss of proliferative potential in dentate gyrus and SVZ stem cells

It has recently been shown that physical exercise is able to fully restore the declined neurogenesis due to the loss of the Btg1 gene in the adult neugenic niches (Farioli-Vecchioli et al., 2014b). It is known, as indicated by several studies, that in the adult dentate gyrus voluntary exercise facilitates both the structural and functional plasticity and enhances cell proliferation and neurogenesis (van Praag et al., 1999 a,b) as well as synaptic plasticity (van Praag et al., 1999b; Farmer et al., 2004; Titterness et al., 2011), thus providing an improvement in several specific hippocampus-dependent behavioural task (Creer et al., 2010; Hopkins et al., 2011; Kohman et al., 2012). Moreover, physical activity is able to overcome in part the depletion of adult neurogenesis occurring during aging and brain disease (Van Praag et al., 2005; Siette et al., 2013; Marlatt et al., 2013).

In the Btg1 knockout mice, 12 days of running significantly increase the cell proliferation and neuroblast differentiation in the adult hippocampal dentate gyrus and SVZ, by reactivating within these adult neurogenic niches the hyperproliferation and expansion of the neural stem cell pool observed only transiently in young Btg1 knockout mice. The events above described appear to be dependent on the running-induced shortening of S-phase and consequently on the cell cycle length of neural stem and progenitor cells. In contrast, in the Btg1 wild-type mice running provokes the shortening of the S-phase and cell cycle only of committed progenitor cells (Farioli-Vecchioli et al., 2014b). Remarkably, these data indicate that the replicative potentiality of the neural stem cells is not limited by aging and that the deprived stem cells pool is still ready to be reactivated through physical exercise if the inhibitory cell cycle control exerted by Btg1 is missing. This also highlights the key role of Btg1 in maintaining the quiescence of adult NSC (Farioli-Vecchioli et al., 2014b; Fig. 1D).

Another recent report suggested that 5 days of voluntary physical exercise do not induce a significant change in cell cycle kinetics of stem/progenitor cells in the dentate gyrus, despite a strong increase of proliferation of newborn neurons in dentate gyrus of runner mice (Fischer et al., 2014). The authors conclude that small cell cycle alterations in cell cycle length after running may represent only a consequence rather than the causal regulating factor of the neural precursor expansion in the dentate gyrus.

It is worth noting that these conflicting data may reflect different experimental paradigms (12 vs 5 days of run as well as different housing conditions of mice during voluntary running). At any rate, they certainly invite a further analysis of the molecular pathways involved in the activation of proliferation induced by running in neural progenitors.

Given the evidence that physical exercise in Btg1-null mice rescues the loss of proliferative capability occurring in older stem cells, this strongly suggests that Btg1 should be examined as a therapeutical target in the process of neural aging.

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