Mol Neurobiol (2012) 46:316–331
DOI 10.1007/s12035-012-8289-2

Driving Apoptosis-relevant Proteins Toward Neural Differentiation

Susana Solá · Márcia M. Aranha · Cecília M. P. Rodrigues

Received: 16 April 2012 / Accepted: 5 June 2012 / Published online: 1 July 2012

Abstract Emerging evidence suggests that apoptosis regulators and executioners may control cell fate, without involving cell death per se. Indeed, several conserved elements of apoptosis are integral components of terminal differentiation, which must be restrictively activated to assure differentiation efficiency, and carefully regulated to avoid cell loss. A better understanding of the molecular mechanisms underlying key checkpoints responsible for neural differentiation, as an alternative to cell death will surely make stem cells more suitable for neuro-replacement therapies. In this review, we summarize recent studies on the mechanisms underlying the non-apoptotic function of p53, caspases, and Bcl-2 family members during neural differentiation. In addition, we discuss how apoptosis-regulatory proteins control the decision between differentiation, self-renewal, and cell death in neural stem cells, and how activity is restrained to prevent cell loss.

S. Solá · M. M. Aranha · C. M. P. Rodrigues
Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL), Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal

S. Solá · C. M. P. Rodrigues
Department of Biochemistry and Human Biology, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal

S. Solá (✉)
Av. Prof. Gama Pinto,
1649-003 Lisbon, Portugal
e-mail: susana.sola@ff.ul.pt

Present Address:
M. M. Aranha
Champalimaud Centre for the Unknown, Lisbon, Portugal

Keywords Apoptosis players · Cell fate · Neural differentiation · Stem cells

Abbreviations

AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Bcl-2	B cell lymphoma-2
CAD	Caspase-activated DNase
CNS	Central nervous system
ESC	Embryonic stem cells
IAP	Inhibitor of apoptosis protein
iPSC	Induced pluripotent stem cells
LTP	Long-term potentiation
MMP	Mitochondrial membrane permeabilization
MSC	Mesenchymal stem cells
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NSC	Neural stem cells
PTM	Postranslational modifications
SVZ	Subventricular zone
TA	Transactivation domain

Introduction

Neurogenesis is the process of generating functionally integrated neurons from progenitor cells. It was traditionally believed to occur only during embryonic stages in the mammalian central nervous system (CNS) [1]; however, almost 50 years ago, pioneering work has suggested the existence of continuing neurogenesis throughout adulthood [2]. Significant progress has been made over the past few years, and investigators have now firmly established that new neurons are indeed born in restricted regions of the adult mammalian

CNS [3–5]. These cells are referred to as neural stem cells (NSC), and are defined by their ability to self-renew and produce all three major CNS cell types, including neurons, astrocytes, and oligodendrocytes. Embryonic stem cells (ESC) and induced pluripotent stem cell (iPSC) lines may constitute an abundant source of NSC that can be further differentiated into a wide variety of functional neurons and glia. iPSC are derived by reprogramming of adult somatic cells into an ESC state [6]. In addition, it has recently been demonstrated that forced expression of a combination of three transcription factors can efficiently convert non-neural human somatic cells into functional induced neuronal cells, another possible source of NSC [7]. Nevertheless, potential medical applications of such cell fate conversion and mechanistic regulation of this process are still in initial stages of investigation.

The discovery of NSC not only provides a unique model system to understand basic mechanisms of neural differentiation, but may also lead to improved strategies for neural tissue repair and cell-based replacement therapies of the nervous system. Although the studies using stem cells are incredibly hopeful, many challenges need to be overcome before clinical application of stem cell-based therapy is successfully adopted in patients with neurological diseases. Namely, it is necessary to understand how to direct and control differentiation of specific target phenotypes required for replacement and repair in each disease, as well as to improve survival and differentiation levels of stem cells after transplantation.

Curiously, it has recently been recognized that cell death-relevant proteins, notably those that operate in the core of the executing apoptosis machinery are functionally involved in differentiation of a wide range of cell types, including neural cells [8–11].

Apoptosis is a highly regulated form of cell death with specific morphological features [12]. In addition, apoptotic cells undergo a number of distinct biochemical events involving loss of mitochondrial membrane potential, DNA fragmentation, and protein cleavage [13]. The apoptotic process may occur by several molecular pathways. The best characterized and most prominent is the intrinsic pathway, involving the mitochondria [14], while the extrinsic pathway is activated by death receptors located at the cellular membrane [15]. Although apparently independent, these two apoptotic pathways may interact through a delicate coordination and cross-talk involving key proteins that are common to both pathways [16, 17].

Most morphological changes of apoptotic cells are caused by cysteine proteases that cleave a restricted group of target substrates after an aspartate residue, and are termed cysteinyl aspartate-specific proteases or caspases [18]. In the death receptor pathway, after interacting with their cognate ligands, death receptors cause the recruitment and oligomerization of the adapter molecule Fas-associating death domain-containing protein (FADD) within the death-inducing signaling complex (DISC). Oligomerized FADD binds the initiator caspase-8 and -10, causing caspase dimerization and activation. This, in turn, will cleave effector caspase-3, -6, and -7, leading to activation of key downstream targets and execution of the apoptotic process [19]. In the mitochondrial pathway, death stimuli may target mitochondria either directly or through transduction by proapoptotic members of the B cell lymphoma-2 (Bcl-2) family, such as Bax and Bak. Indeed, mitochondrial membrane permeabilization (MMP) may occur by the opening of the mitochondrial permeability transition (MPT) pore, or through the formation of specific release channels in the mitochondrial outer membrane, promoted by proapoptotic members of the Bcl-2 family. Once induced, MMP causes cytochrome *c* release from mitochondria. Cytosolic cytochrome *c* then induces olimerization of Apaf-1 protein that recruits and activates procaspase-9, which will activate effector caspases, and subsequently cleave other cellular substrates [20]. In the mitochondria-mediated apoptotic pathway, Bcl-2 family members play a key role in integrating survival and death signals ultimately regulating MMP. Bcl-2 family proteins can be categorized into different groups, according to their structure and function, and are capable of interacting with and inhibiting each other. The balance of pro- and anti-apoptotic members of the Bcl-2 family at the mitochondrial membrane is crucial to modulate MPT pore opening, cytochrome *c* release, and subsequent nuclear fragmentation [21]. For instance, many pro-apoptotic members of the Bcl-2 family play an important role in apoptosis induced by tumor suppressor protein p53 [22, 23]. In fact, p53 is critical in the regulation of Bcl-2 family proteins, antagonizing the anti-apoptotic function of Bcl-x_L or Bcl-2 [24, 25], through both transcription-dependent and -independent mechanisms. Moreover, cytoplasmic p53 can physically interact with members of the Bcl-2 protein family, thereby promoting MMP [26].

Nevertheless, a clear consensus about the mechanisms by which components of apoptosis may also promote differentiation, and do not entail the induction of cell death is still missing. Once identified, these mechanisms could be strategically manipulated to improve neural differentiation as an alternative to cell death, bringing stem cells one step closer to successful clinical application. In fact, although active apoptotic events might be tightly regulated to avoid cell death, some differentiating cells might still end up dying instead of proceeding to neural differentiation. Here, we discuss the potential role of apoptosis-relevant proteins in neural differentiation and elucidate key checkpoints, which will accelerate efforts to generate clinically relevant cell types from stem cells.

Apoptosis-relevant Players in Neural Differentiation

The regulation of cell number is a crucial property of complex multicellular eukaryotes. Evolution has selected for the development of elegant mechanisms to modulate rates of both cell birth and cell death. In this respect, apoptosis can be viewed as a process that eliminates superfluous, ectopic, damaged, or mutated cells according to the rule "better death than wrong" [20]. In fact, apoptosis is a highly regulated form of cell death known to sculpt tissues during development and maintains tissue homeostasis by eliminating unnecessary or harmful cells [12]. However, apoptosis is also thought to result from a failure of cells to correctly exit the cell cycle and differentiate. Several compounds of the apoptotic machinery become activated during, and are required for, the differentiation process. These molecules might function as the molecular switch that drives cell fate [27].

Next, we will discuss the emerging evidence for non-apoptotic functions of components of the apoptotic apparatus in mediating neural cell fate specification and differentiation.

The Tumor Suppressor p53

p53 is a tumor suppressor protein that induces cell cycle arrest, senescence, or apoptosis in response to cellular stresses. Owing to its ability to integrate many different signals controlling cell life and death, p53 has been named the "guardian of the genome" and plays a crucial role in maintaining genomic stability in somatic cells [28]. p53 functions mainly as a DNA-binding, sequence-specific transcription factor. Most evidence suggests that the key contribution of p53 to apoptosis is its ability to activate the transcription of various pro-apoptotic genes, including members of the Bcl-2 family, such as *bax*, *noxa*, and *puma* [23, 29]. p53 also activates the expression of multiple genes, including *p21* [28], which in turn mediate cell cycle arrest and senescence.

The capacity of p53 to maintain genomic stability seems restricted to somatic cells. Recent studies suggest the involvement of p53 in the maintenance of genomic stability in developing cells by inducing cell differentiation, a potentially new cellular outcome. In fact, the ability of ESC to differentiate into multiple cell types requires a highly sensitive and finely tuned p53-dependent signal transduction pathway to cope with DNA damage, which otherwise could promote tumorigenesis and pass mutations to off-spring cells. Accordingly, it has been shown that the rate of spontaneous mutation in ESC is significantly lower when compared with somatic cells [30]. Nevertheless, it has been demonstrated that p53-mediated cell cycle checkpoint is compromised in mouse ESC [31]. Others have revealed that p53 maintains genetic stability of ESC by inducing differentiation. In fact, following genotoxic stress, p53 induces differentiation of ESC by directly suppressing *Nanog* expression [32]. Using genome-wide approaches, it was demonstrated that p53 may affect the ESC status by acting not only on repression of ESC-enriched genes, but also by activating differentiation-associated genes [33]. Therefore, the induction of ESC differentiation by p53 constitutes an alternative mechanism to maintain genetic stability. Once differentiated into other cell types, these cells can undergo efficient p53-dependent cell cycle arrest or apoptosis.

Interestingly, p53 was also shown to suppress *Nanog* expression after treatment with retinoic acid, corroborating the idea that p53 is important not only to maintain genomic stability but also to differentiate ESC. Thus, p53 should be viewed as a crucial decision-maker molecule, rather than a tumor suppressor protein [28, 34]. In fact, p53 knockdown reduced spontaneous differentiation and slowed directed differentiation rate in human ESC [35], while activation of p53 by the small-molecule activator nutlin led to rapid differentiation [36]. In addition, a novel role has recently been identified for natriuretic peptide receptor-C (NPR-C) in the survival of murine ESC through mechanisms dependent on p53 [37]. NPR-C was required to control DNA damage-induced p53 levels and maintain ESC self-renewal.

p53 plays a well-established role in protecting neurons from exposure to a range of stressors and DNA damage through the induction of senescence, DNA repair, and apoptosis [34]. Indeed, p53 has a crucial role in eliciting neuronal cell death during development [34]. Although much still needs to be learned, recent evidence supports exciting new roles for p53 in a wide range of mechanisms, including neural precursor cell self-renewal, differentiation, and cell fate decisions. It will be crucial to understand the full repertoire of p53 function in cell proliferation and differentiation of NSC, in particular the posttranslational modifications (PTM) that allow p53 to interact with distinct cellular targets to induce a specific outcome in neural differentiation.

Finally, p53-related p63 and p73 proteins share considerable structural and functional homology with p53. These proteins, encoded by the *Trp63* and *Trp73* genes, are also transcription factors involved in the regulation of development, cell death, proliferation, stem cell renewal, cell fate commitment, as well as tumorigenesis [38, 39]. Mouse gene targeting studies have revealed that both *Trp63* and *Trp73* are required for normal embryogenesis [40–42]. p63 and p73 are important regulators of cell survival and cell death in the nervous system, primarily by acting through the apoptosis machinery [43–45]. p63 function in sympathetic neuronal development is firmly established [45]; however, two independent studies have recently reported that p63 may not be essential for CNS development [46, 47].

Nevertheless, in contrast to p63-restricted expression observed in mice and human brain development, high levels of p63 protein and mRNA were observed in adult human cerebral cortex and hippocampus [46], suggesting a role for p63 in the adult brain. Furthermore, in postnatal mice, p63 and p73 are highly expressed in a neurogenetic niche, such as the subventricular zone of the lateral ventricle [46]. Interestingly, the pro-neurogenic function of p73 has also been further characterized in the past years.

p53 in Neural Differentiation Before the link of p53 and *Nanog* had been established, mounting evidence was already suggesting a role for p53 in neuronal differentiation (Fig. 1). Initially, p53 was considered not essential for embryonic development, since the majority of p53-null mice were considered phenotypically normal at birth, with markedly increased tumor development later in life [48]. However, the discovery that a fraction of p53-deficient embryos do not develop normally and exhibit neural tube defects [49, 50] was an apparent paradox, and suggested that the role of p53 in development may be more complex than initially believed. p53 mRNA levels during mouse embryonic development peak during differentiation of several tissues, including early neuronal precursor cells of the brain, but strongly declines during terminal differentiation [51–53]. In addition, evaluation of p53-dependent transcriptional activation during normal development in vivo indicated that p53 activity is at maximum during neuronal differentiation, clustering in areas that are not correlated with apoptosis [54, 55]. Later work has gradually supported a link between p53 and neural differentiation. In vitro studies of neuronal differentiation, using neuronal-like PC12 pheochromocytoma and neuroblastoma cells revealed that p53 gene expression is induced and required during neurotrophin-dependent neuronal differentiation and maturation [56–59]. In fact, p53 was reported to play a critical role in nerve growth factor (NGF)-mediated neuronal differentiation of PC12 cells, in part via regulation of tropomyosin-related kinase A (TrkA) expression, which in turn activates mitogen-activated protein kinase (MAPK) pathways [59, 60]. Moreover, new putative p53 target genes have been recently identified by genome-wide chromatin immunoprecipitation during NGF-mediated PC12 neuronal differentiation, among which are *wnt7b* involved in dendritic extension and the *tfcp214/grhl3* grainyhead homolog implicated in ectodermal development [61]. p53 regulates the expression of both actin-binding protein Coronin 1b and the GTPase Rab13 in primary cultured neurons, both of which associate with the cytoskeleton and regulate neurite outgrowth [62], (Fig. 1). However, recent studies using chromatin-immunoprecipitation-based microarrays coupled with gene expression microarrays revealed that p53 has an anti-differentiation function in mouse ESC through direct regulation of the Wnt signaling pathway [63]. This observation is inconsistent with the well-established pro-differentiation role of p53 and may be related with different genetic backgrounds of mouse ESC.

Much like in PC12 cells, p53 has been also shown crucial for correct neurite outgrowth and maturation of cortical neurons [64, 65]. After neuronal injury, p53 PTM may protect neurons from stress and DNA damage through the induction of cell cycle arrest. This leading role of p53 contributes to directing neurons toward a specific phenotype in critical conditions, such as during development and following cellular damage [66]. Recently, several studies have extended the knowledge of the precise mechanism of action of p53 during neural differentiation. p53 was revealed to interfere with the Akt/p-FOXO3A/Id1 survival pathway [67] by downregulating this signaling pathway during the neural differentiation process. Interestingly, p53 activity increases mainly during neurogenesis, and p53 silencing leads to a delay in neurogenesis but not in gliogenesis. Importantly, the increase in p53 activity was not associated with cell death in this specific cellular model. The role of p53 during mouse NSC differentiation has been further dissected by other studies [68, 69]. New molecular links between p53 and key regulators of neurogenesis, such as the dual oxidase maturation factor 1 [68], and the histone H3 lysine 27-specific demethylase JMJD3 [69] were also identified. Interestingly, the cross-talk between p53 and JMJD3 during mouse neural differentiation appears to include p53 stabilization and demethylation by JMJD3. This, in turn, results in p53 nuclear accumulation during specific stages of neural differentiation.

Despite the supporting data on transcriptional activity of p53 protein in neural differentiation, the precise mechanisms through which p53 acts in this cellular context is still a puzzling question, often associated with its regulation of cell cycle progression.

p53 as a Limiting Factor of Cell Proliferation Several observations have demonstrated that p53 might play a key role in neural differentiation as a limiting factor of stem cell proliferative competence [70, 71]. In fact, the demonstration that p53 is expressed in proliferating and newly formed neurons of the embryonic and postnatal rat brain and that its expression is associated with cell cycle regulators such as p21 [72] was the first indication that p53-dependent molecular pathways may affect neural progenitor cell proliferation and therefore neural differentiation. p53 was reported to act as a negative regulator of NSC self-renewal [73]. It has subsequently been shown that p21, an established mediator of p53-dependent cell cycle arrest, can negatively regulate the self-renewal of adult NSC [74]. In addition, the absence of p53 protein was shown to increase the proliferation of

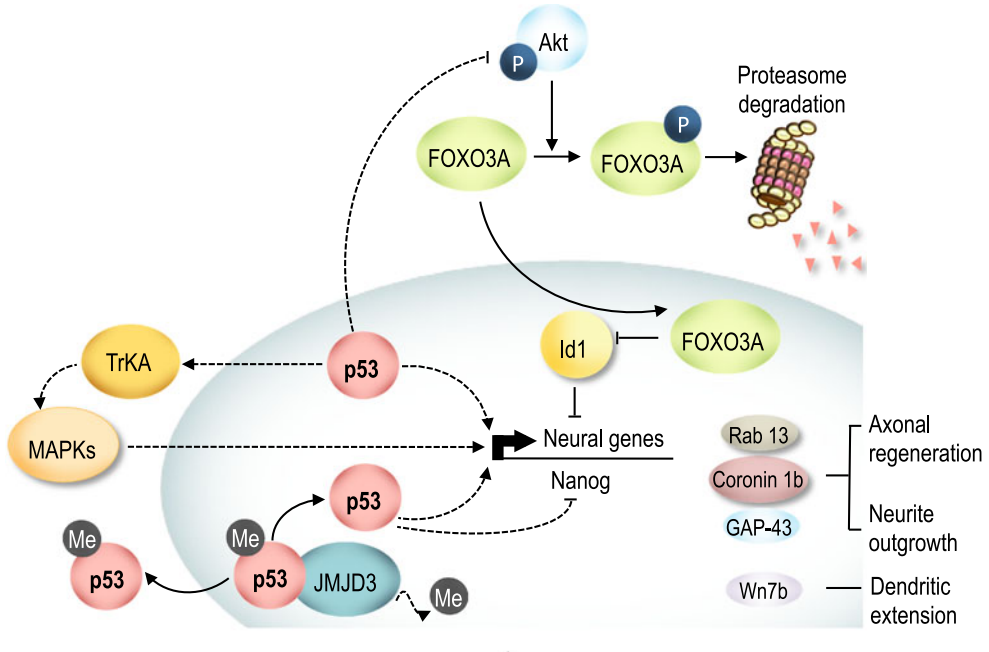

Fig. 1 Proneurogenic p53 regulation mechanisms and physiological functions. p53 is dynamically involved in nonapoptotic processes such as cell-fate determination and actin-cytoskeleton reorganization. p53 plays a critical role in neuronal differentiation, in part via regulation of tropomyosin-related kinase A (*TrkA*) and mitogen-activated protein kinase (*MAPK*) pathways in PC12 cells. Moreover, Wnt7b was recently identified as a new putative p53 target during nerve growth factor (*NGF*)-mediated PC12 neuronal differentiation, involved in dendritic extension. p53 also regulates the expression of actin-binding protein Coronin 1b and the GTPase Rab13 in primary cultured neurons, both of which associate with the cytoskeleton and regulate neurite outgrowth, as well as the expression of the axonal growth-associated protein GAP-43, a well-characterized pro-axon outgrowth and regeneration protein. In mouse neural stem cells, p53 promotes neurogenesis by downregulating the Akt/p-FOXO3A/Id1 proliferation pathway. Interestingly, the histone H3 lysine 27-specific demethylase JMJD3, a key regulator of neurogenesis, directly interacts with p53 to induce demethylation of p53 and subsequent p53 nuclear accumulation during specific stages of neural differentiation

stem cells derived from the olfactory bulb [71], while PC12 cells containing mutant p53 were not able to cease proliferation and begin neuronal differentiation following NGF treatment [75]. However, a positive correlation has been shown between cell density and neuron percentage, with enrichment of neurosphere-forming cells in p53-null mice embryos [71]. Although loss of p53 by itself is not sufficient for tumor formation, it can provide a proliferative advantage to the slow- and fast-proliferating subventricular zone (SVZ) stem cell populations, associated with their rapid differentiation [76]. Loss of p53 was strongly related with increased number of adult NSC and neuroblasts. More recently, the role of p53 in regulating cell proliferation in the murine adult SVZ zone has been characterized, where p53 knockout resulted in higher levels of NeuroD and Math3 and generated neurons more readily. In this study, p53 and p27 function in a non-redundant manner to modulate proliferation and self-renewal, and in antagonist fashion to regulate adult neurogenesis [77]. Curiously, it has also been demonstrated that the orphan nuclear receptor TLX acts as an essential regulator, which ensures the proliferative ability of postnatal NSC by controlling activation through genetic interaction with p53 signaling pathways [78]. In fact, NSC continually produce new neurons in postnatal brains; however, the majority of these cells stay in a nondividing, inactive state and the molecular mechanism required to enter proliferation remains largely unknown. TLX regulates p21 expression in a p53-dependent manner and, more importantly, acute removal of p53 rescues the proliferation defect of TLX-null NSC in culture. Therefore, the precise role of p53 during neurogenesis remains to be further elucidated, namely whether neurogenesis induced by the absence of p53 is only the ultimate result of increased number of neural progenitors. Notably, p53 was not essential to promote cell death in any of these studies, which supports the idea that p53 is required to maintain the physiological proliferation rate in NSC.

Switch of p53 Biological Function Genomic elements regulated by p53 during neuronal differentiation may be unique, and different from those regulated in genotoxic stress and apoptosis. This is made possible by the multiple PTM that target p53 on its N- and C-termini, which are not limited to phosphorylation and may also include acetylation, sumoylation, neddylation, and ubiquitination [79, 80]. It is now clear that p53 PTM directly affect the transcriptional

activity of p53 and regulate its affinity to diverse cofactors, which in turn regulate the occupancy of p53-specific promoters [81]. Nevertheless, the functional relevance of specific PTM and how it influences the occurrence of additional PTM and protein-protein interactions is still unknown. Importantly, soon after the onset of ESC differentiation, phosphorylation of p53 at Ser15, Ser315 and Ser392 is significantly increased, which is thought to activate p53 [82]. p53 phosphorylation at Ser315 was also shown to be particularly important for p53-dependent suppression of *Nanog* during differentiation of ESC [32]. The acetylation of p53 involves the activity of at least two histone acetyltransferases, CREB-binding protein (CBP)/p300 and p300/CBP-associated protein (P/CAF). Of note, during neuronal differentiation and axon outgrowth, kinase and acetyltransferase pathways were shown to be activated downstream from NGF and brain-derived nerve growth factor signaling. Interestingly, the acetylation of p53 at Lys320 results in increased transcriptional activation of *p21* and subsequent cell cycle arrest, associated with neuronal differentiation in PC12 cells [83]. p53 acetylation in the same residue was reported to be involved in promotion of neurite outgrowth [64]. In addition, acetylated p53 at Lys372, Lys373, and Lys382 was reported to drive axon outgrowth and growth associated protein-43 (GAP-43) expression, and to bind specific elements on neuronal GAP-43 promoter [65].

On the basis of our current knowledge of how specific PTM affect p53 activity, it is tempting to speculate that functional interference between specific acetylated and phosphorylated residues of p53 may also dictate cell fate. For instance, acetylation of Lys320 prevents phosphorylation of crucial serines in the N-terminal region of p53, which only allows the activation of genes that contain high-affinity p53 binding sites, such as *p21*. On the other hand, acetylation of Lys373 results in hyperphosphorylation of p53 N-terminal residues and enhances the interaction with promoters for which p53 possess low DNA binding affinity [84]. Therefore, a specific combination of PTM differentially regulates the interaction of p53 with co-activators and co-repressors and produces distinct gene-expression profiles.

p53-induced control of cell fate may also be associated with p53 subcellular distribution. In fact, it has been reported that p53 translocates to the nucleus following induction of differentiation [85, 86]. SIRT1, a p53 deacetylase, negatively regulates p53 nuclear translocation in mouse ESC and inhibits p53-mediated suppression of *Nanog* expression [75]. Moreover, p53 demethylation is implicated in p53 nuclear localization during mouse NSC differentiation, associated with an increase in neuronal markers [69]. In fact, recent studies have already established protein methylation as a novel mechanism of p53 regulation [87]. Several histone lysine methyltransferases have been shown to methylate p53 at specific C-terminal lysines, preventing or inducing p53 interaction with its co-activators. p53 directly interacts with lysine-specific demethylase 1 (LSD1) to alter chromatin structure and confer developmental repression of a specific tumor marker [88]. On the other hand, the ability of p53 to modulate different transcriptional responses in neurons, depending on specific signaling and transcriptional contexts might be linked not only to the PTM of p53, but also to the cross-talk between p53 and PTM on histones. Histone modifications influence chromatin structure, which in turn influences the capacity of transcription factors to bind to specific promoters. A unique chromatin environment could indeed regulate the affinity of p53 to specific target promoters. Studies in neuronal cells have indicated that the interaction of p53 with the neuronal-specific and pro-differentiation transcription factor Brn-3a facilitates a shift of p53 transcriptional activity from cell death to neuronal differentiation [89]. When forming a complex with Brn-3a, p53 is not able to activate pro-cell death genes such as *Bax* and *Noxa*, but rather shows increased affinity for the pro-differentiation gene *p21*. In this respect, Brn-3a might play a crucial role in determining the pathway taken by p53, when co-expressed during development, and in controlling cell fate. p53 may generally contribute to neuronal differentiation by targeting cell cycle regulators, and particularly by controlling the expression of differentiation-specific genes in a cell-type-specific manner. This could be directed by the interaction between Brn-3a and p53, which facilitates a shift of p53 transcriptional activity from cell death to neuronal differentiation. Further, p53 activity may be regulated through repression of mRNAs involved in pathways regulating p53. Such posttranscriptional regulation has been described for spermatogenesis, in which mRNAs encoding activators of p53 were repressed by Pumilio 1 [90].

p53 may be considered a gatekeeper of self-renewal in NSC that may function either as pro-apoptotic protein or differentiation factor in neural precursors. Specific patterns of p53 codes might lead to different biological outcomes, depending on the transcriptional context in a given cell or tissue [91]. Understanding the role of such mechanisms in the regulation of the neuronal phenotype during development undoubtedly represents an exciting challenge for future investigation.

p73 in Neural Differentiation Discovered in 1997 [92], the *Trp73* gene contains two promoters that drive the expression of two major groups of p73 isoforms with opposing cellular actions. The TAp73 isoforms contain the p73 transactivation domain (TA) and exhibit proapoptotic activities [93], whereas the ΔNp73 isoforms lacking the N-terminal TA domain are anti-apoptotic [94].

The first evidence revealing unique roles for p73 in neurogenesis came from the generation of knock-out mice

[42]. Mice functionally deficient for all p73 isoforms exhibit profound defects, including hippocampal dysgenesis, hydrocephalus, chronic infections, and inflammation, as well as abnormalities in pheromone sensory pathways. Curiously, dominant-negative p73 variants are the predominant expression product of the p73 gene in developing and adult tissues. The role of p73 in neural differentiation was later confirmed by the generation of the isoform selective knockout mice [95]. Mice specifically lacking the TAp73 isoforms develop a phenotype intermediate between the phenotypes of *Trp73*−/− and *Trp*53−/− mice with respect to incidence of hippocampal dysgenesis. TAp73 isoforms exert tumor-suppressive functions, indicating an important role for *Trp73* in the maintenance of genomic stability. The role of p73 in neural differentiation has been also characterized [96], where the p73 isoforms activate a molecular pathway that is not shared by p53 and is required for neuroblastoma cell differentiation in vitro. In fact, endogenous p73 levels increase in neuroblastoma cells induced to differentiate by retinoic acid. Exogenously expressed p73, in turn, is sufficient to induce neurite outgrowth, expression of neurofilaments and neural adhesion molecule (N-CAM), down-regulation of N-Myc, and up-regulation of pRb and all markers of neural differentiation. Nevertheless, the first evidence for the role of p73 in differentiation of a normal mammalian cell came from studies showing that both p53 and p73, but not p63, are involved in thyroid hormone-induced oligodendrocyte precursor cells differentiation, and that p73 also plays a crucial part in platelet-derived growth factor (PDGF) withdrawal-induced differentiation [97]. More recently, several studies have demonstrated that p73, in particular the TA isoform, is an essential regulator of stemness in NSC. p73 maintains an adequate neurogenic pool by promoting self-renewal and proliferation and inhibiting premature senescence of neural stem and early progenitor cells in both embryonic and adult neurogenesis [98]. NSC from p73 null mice have a reduced proliferative potential, together with reduced expression of members of the Sox-2 and Notch gene families, known to be important for NSC proliferation. In addition, the width of neurogenic areas is significantly reduced in the brains of embryonic and adult p73 knockout mice [99]. Curiously, TAp73 appears to act via the basic helix-loop-helix (bHLH) Hey2 to promote long-term maintenance of neural precursors [100]. TAp73 transcriptionally regulates bHLH Hey2, which itself promotes neural precursor maintenance by preventing premature differentiation. Further, p73 deficiency results in impaired self-renewal and premature neuronal differentiation of mouse neural progenitors independently of p53 [101]. A comparative study of neurospheres from *Trp73*−/−, *Trp53*−/−, and *Trp53*−/− and *Trp73*−/− and their wild-type counterparts demonstrated that p73 deficiency results in two independent, but related phenotypes: a smaller neurosphere size, related to proliferation and survival of neural-progenitors, and a decreased capacity to form neurospheres. The former results from p53 compensatory activity, whereas the latter is p53 independent. Finally, p73 drives the expression of microRNA-34a in mouse cortical neurons, which in turn modulates the expression of synaptic targets, including synaptotagmin-1 and syntaxin-1A [102]. Retinoid-driven neuroblastoma differentiation is inhibited by knocking down p73 or miR-34, while miR-34a and p73 are increased during postnatal development of the brain and cerebellum, when synaptogenesis occurs [103].

Caspases

Caspases are cysteinyl aspartate-specific proteases with a number of properties that make them highly effective as cellular remodeling enzymes. Caspases are conserved in multicellular organisms and function as central regulators of apoptosis. Although their critical function in apoptosis is firmly established, caspase activation can also indicate the onset of differentiation, inflammation, and other vital processes. In fact, caspases are a multifunctional, highly regulated family of enzymes that catalyze a biologically diverse set of reactions. Members of the caspase family are subdivided into upstream "initiator" caspases (in mammals, caspase-1, -2, -4, -5, -8, -9, -10, -11, and -12), which respond to pro-apoptotic signals, and downstream "executioner" caspases (in mammals, caspase-3, -6, -7, and -14) (for a review, see [104, 105]). Following activation, initiator caspases cleave executioner procaspase-3, -6, and -7, which in turn leads to cleavage of more than 1,000 proteins [106, 107], nearly 5 % of the cellular proteome. The first caspase to be characterized, caspase-1, is associated to inflammatory processes [108]. Therefore, it is not surprising that some caspases mediate non-apoptotic functions. We are only beginning to understand caspase function in other cellular remodeling events, and it is clear that caspases are more than just harbingers of cellular death. Curiously, recent data on non-apoptotic functions of caspases have provided more evidence for possible existence of a DNA breakage mechanism in differentiating cells resembling the initial stage of apoptosis [109]. Caspase signals often result in the activation of the specific nuclease caspase-activated DNase (CAD). Recently, it has been shown that caspase-3/CAD promotes cell differentiation by directly modifying the DNA/nuclear microenvironment, which enhances the expression of critical regulatory genes [110]. In this regard, caspases induce directed DNA damage, which in turn may participate in numerous forms of regulated gene expression throughout differentiation [111]. On the other hand, the role of caspases has been well established during terminal

differentiation of a wide range of cell types such as megakaryocytes, erythrocytes, keratinocytes, and epithelial cells in the lens [112–115]. The enucleation process of lens epithelial cells is prevented after inhibition of caspase activity by the pan caspase inhibitor, z-VAD.fmk, suggesting that caspases are involved in the specific elimination of nuclei as opposed to elimination of the whole cell [116]. Moreover, two recent studies implicate caspase-3 in controlling fate of both ESC and adult hematopoietic stem cells. One study reported caspase-induced cleavage of Nanog in differentiating ESC [117]. Unsurprisingly, stem cells lacking the *caspase-3* gene showed marked defects in differentiation, while forced expression of caspase cleavage-resistant Nanog mutant in ESC strongly promoted self-renewal. This study has revealed Nanog as a key caspase substrate for limiting the self-renewing capacity of ESC. Curiously, caspase-3 has been shown to contribute to stem cell quiescence, by dampening specific signaling events. Alteration in caspase-3 expression perturbs homeostasis of primitive hematopoietic cells [118], while caspase-3-deficient stem cells display accelerated proliferation and retarded differentiation. At the molecular level, caspase-3 appears to alter the sensitivity of primary hematopoietic cells to cytokine stimulation through modulation of specific signaling pathways, such as extracellular signal-related kinase (ERK) signaling. Caspase-3 was also shown to be implicated in differentiation of other cell types, including osteoclasts [119], bone marrow stromal cells [120], and skeletal muscle cells [8]. Interestingly, caspases have recently been identified as key facilitators of nuclear reprogramming in iPSC induction. In addition, the retinoblastoma (Rb) susceptibility protein appears to act downstream of caspases [121]. Collectively, these data suggest that during cell remodeling, caspases cleave but not always degrade their substrate, thus modulating protein function and localization [122].

Caspases in Neuronal Differentiation Genetic manipulation of caspase activity in *Drosophila* and mice has revealed that caspases control cell fate through apoptotic and non-apoptotic mechanisms, to ensure appropriate cell differentiation and maturation in the developing nervous system. In fact, caspases are essential not only for controlling the number of cells involved in sculpting or deleting structures in neural tissues, but also for dynamic, non-apoptotic cell processes, such as innate immune response, neural regeneration, NSC-fate determination, NSC differentiation and neural activation. In *Drosophila*, caspases are required for dendritic pruning in sensory neurons [123]. Pruning is a process often used to selectively eliminate excessive neuronal projections, including axons, dendrites and synaptic connections, without the death of parent neurons. In fact, mutations in either the initiator caspase Dronc [124], the apical caspase Dredd, or the effector caspase Drice, all failed to attenuate this type of pruning. In addition, caspase-3 activity is regulated at postsynaptic sites in the brain following stimuli associated with memory instead of cell death. A recent study on response habituation in adult zebra finches has provided direct evidence for caspase-3 activation by animal training [125]. Following exposure to tape-recorded birdsong, the concentration of the active form of caspase-3 sites are increased within minutes in dendritic spines in the auditory forebrain. Interestingly, activated caspase-3 was present even in unstimulated brain but bound to an endogenous inhibitor of apoptosis proteins (IAP), BIRC4, suggesting a mechanism for rapid release and sequestering at specific synaptic sites. Although nothing is known about the mechanism by which caspase-3 affects learning and memory, it may involve cleavage of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and thereby reduction in glutamatergic responses and prolong spike habituation [126]. In fact, the GluR1 subunit of the AMPA subtype of glutamate receptors is directly cleaved by caspase-3, and cleavage of this subunit modulates neuronal excitability in ways that suggest important roles for caspases in regulating synaptic plasticity and cell survival. Consistent with caspase-dependent memory consolidation in zebra finches, a recent study have also shown that synaptic depression and AMPA receptor internalization in mouse hippocampal neurons requires caspase-9 and caspase-3/7 activity, which is blocked by overexpression of anti-apoptotic proteins Bcl-x_L and XIAP [127]. Stimulating *N*-methyl-D-aspartic acid or *N*-methyl-D-aspartate (NMDA) receptors transiently activates caspase-3 without causing cell death. Moreover, long-term synaptic depression cannot be induced by stimulating NMDA receptors in hippocampal slices from caspase-3 knockout mice [127]. In addition to long-term depression (LTD), long-term potentiation (LTP) seems to be also a critical caspase-mediated process influencing learning and memory. However, the role of caspases in mediating LTP has been controversial. Inhibition of caspase-3 significantly decreases or fully blocks LTP in the CA1 region, suggesting that caspase-3 is essential for LTP [128]. Nevertheless, Aβ-inhibited hippocampal LTP was shown to occur through a signaling pathway involving caspase-3 [129]. This idea, in turn, is in agreement with the previous observation showing that inhibition of caspase-3 prevents the structural rearrangements in LTP associated with the involvement of new synapses and neurons in the response [130].

Interestingly, in the eye imaginal disc, after apoptotic stimulation, effector caspases in photoreceptor neurons stimulate Hedgehog (Hh) signaling, thereby triggering normally post-mitotic cells to reenter the cell cycle [131, 132]. The cell autonomous pro-differentiation effects of caspases have also been reported in PC12 cells [133] and in neural and glial progenitor cells [9, 134]. Non-apoptotic active

caspase-3 expression has been shown to be located in proliferating and differentiating neuronal cells of the SVZ and external granular layer of the developing cerebellar cortex [134]. Interestingly, neural differentiation of both PC12 cells and neurospheres has also been shown to require transient caspase activation [9, 133]. In fact, caspase activation is not sufficient to cleave enough death substrates to execute apoptosis. Non-apoptotic caspase function in neural development has been further elucidated by others [135, 136]. Caspase-3 activity is required for growth cones in cultured retinal cells to respond to the chemotropic molecules netrin-1- or lysophosphatidic acid (LPA) [135]. In addition, caspase-9- and Apaf-1-mutant mice exhibit misrouted axons, impaired synaptic formation, and defects in olfactory sensory neuron maturation [136]. These observations underscore a conserved relevant role of caspases in regulation of neural differentiation, as indicated in Fig. 2a. Now that cataloging substrates is becoming routine, it is time to focus on how caspases distinguish their substrates and cleavage sites in the complex mixture of cellular proteins.

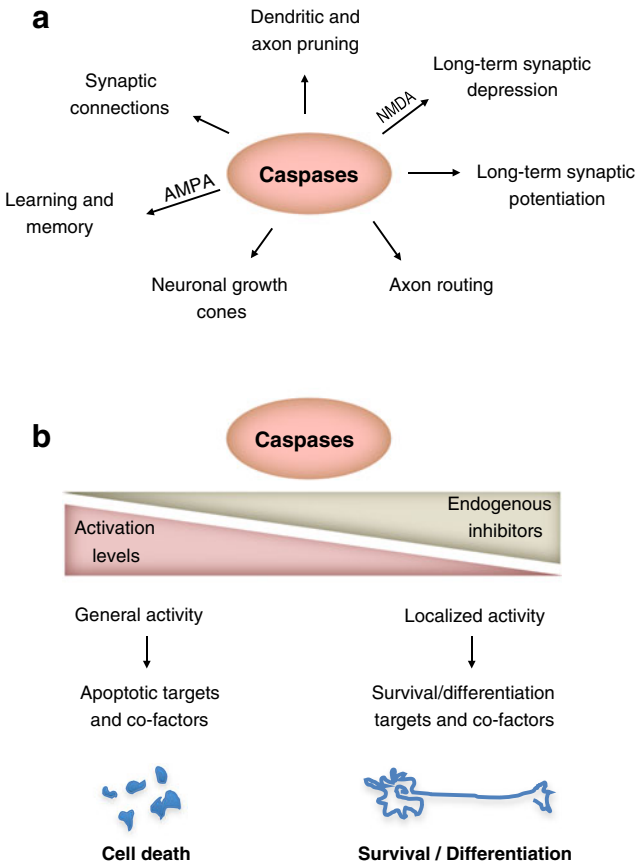

Fig. 2 Non-apoptotic role of caspases in neuronal cells. **a** Schematic diagram of different cellular outcomes of caspase-mediated non-apoptotic mechanisms. **b** Possible mechanisms have been proposed to control active caspases without compromising cellular integrity. The specific outcome may depend on the extent and/or kinetics of caspase activation, sub-cellular localization of activated caspases, and also on expression levels of endogenous caspase inhibitors, such as inhibitor of apoptosis protein (IAP). In addition, caspases may induce differentiation, as an alternative to cell death, by interacting with specific targets and co-factors. In fact, caspases may actively engage factors that promote the gene expression and phenotype of a differentiated cell type, in addition to limiting self-renewal, hence favoring neural differentiation

Switch of Caspase Biological Function It is still unclear how caspases are activated in non-apoptotic processes and how differentiating cells restrain active caspases without compromising cellular integrity. Several possible mechanisms for regulating non-apoptotic caspase function have been proposed, including transient caspase activation, local caspase activation and regulation of endogenous caspase inhibitors. For example, transient caspase activation regulates sensory bristle differentiation. It has been shown that the precise temporal control of *Drosophila* inhibitor of apoptosis protein 1 (DIAP1) is critical for maintaining the balance between cell viability and execution of caspase non-apoptotic functions [137]. Indeed, in the absence of substrate selectivity, a plausible explanation for death versus differentiation phenotypes may originate with the timing and intensity of signal pathway activation, as illustrated in Fig. 2b. Specifically, the degree of caspase activity required to effectively act on differentiation signals might be lower than that observed to induce apoptosis [138]. This specific reduction on caspase activity was shown to be crucial during NMDA-dependent LTD, a process in which caspase-3 plays a crucial role [139]. In this study, the authors demonstrated that caspase-3 is activated by Bad-Bax signaling, in a similar way than in apoptotic contexts. However, in contrast to apoptosis, Bad is only moderately and transiently activated, and Bax is not translocated to mitochondria, resulting in modest caspase-3 activation and non-lethal cellular outcome. In contrast, other studies support a mechanism in which active caspase-3 is always present in living cells, but is sequestered by its inhibitors and released only transiently for essential non-apoptotic functions, including caspase-mediated neural activation [125]. The extent of caspase-3 activation may be determined by upstream regulators, which in turn dictate cell death or other cellular fate.

Other explanation is that the choice between death and differentiation derives largely from caspase targeting and activation of specific substrates or co-factors that are unique to each event. In this regard, it has been shown that during differentiation, but not during apoptosis, the chaperone protein Hsp70 protects the transcription factor GATA-1, a master regulator of erythroid maturation, from caspase-mediated proteolysis during the enucleation process of erythroids [140]. It is unclear, however, whether the selective masking of caspase substrates by Hsp70 provides a universal control mechanism for substrate specificity. Clearly, a systematic investigation of each caspase substrate based on a proteomics approach is needed to clarify

whether caspases maintain a regulatory role in both apoptosis and cell differentiation.

In addition to temporal kinetics and substrate selectivity, the final choice between death and differentiation may also depend on subcellular localization of activated caspases and their constituent regulatory pathways. Localized caspase activity has been found to play an important role during sperm development. In fact, dramatic removal of bulk cytoplasm occurs in terminal differentiation of spermatid into individual sperm. Caspase-3 is detected in the individualization complex, a cytoskeletal membrane complex that moves along the length of the cyst to the sperm tail [141]. In this case, caspase activation is restricted to areas far away from the soma, and a cytoplasmic death substrate such as the inhibitor of CAD might not be effectively cleaved. Finally, the specific subcellular localization of activated caspases might be strictly linked to the presence of inhibitory proteins of IAPs [142]. It has been suggested that binding of active caspase-3 to an endogenous inhibitor can be a mechanism for rapid release and sequestration of caspase-3 at specific sites [125]. Caspase-3 active sites increase transiently in songbird auditory forebrain during song habituation training. In addition, caspase-3 is present in dendritic spines of unstimulated brain, but in an inactive state through interaction with endogenous inhibitor X-linked inhibitor of apoptosis protein (XIAP). In fact, mechanisms to control caspase activation are essential for maintaining cell integrity, and IAPs are responsible for much of this control. More recently, it was shown that regulation of the non-apoptotic function of caspases during spermatogenesis relies on the ubiquitin proteasome system that targets IAP-like proteins at specific sites for local degradation, releasing caspases from inhibition [143, 144]. This provides a mechanism to explain how spatial regulation of caspases can be achieved to drive differentiation instead of cell death execution [143]. Accordingly, it has been reported that cell differentiation in neurons is accompanied by a decrease in Apaf-1 and activity of the apoptosome with an increased ability of IAPs to sustain survival [145]. The activity of the apoptosome changes with neuronal differentiation with alterations in both Apaf-1 and efficacy of IAPs. Further, during differentiation of PC12 cells and primary sympathetic neurons, levels of Apaf-1, and hence the apoptosome, are reduce and protection by IAPs is increased. In fact, Apaf-1 levels are high in non-differentiated PC12 cells and sympathetic neurons, but drop dramatically during neuronal differentiation. This renders mature neurons resistant to cytochrome *c*-triggered apoptosis. Although no changes in IAPs or caspase-9 were reported, Apaf-1 levels and apoptosome activity were reduced, allowing for more effective regulation by IAPs. Consistent with this outcome, it has been shown that differentiated PC12 cells have increased resistance to cell death through an IAP-dependent mechanism [146]. In contrast, the stemness cell marker Nanog was shown to be pivotal in upregulating IAPs in neck squamous cell carcinoma [147], indicating that pluripotency may be also correlated with increased IAP levels. Collectively, these studies indicate that multiple mechanisms may act synergistically, in a cell type-dependent manner to restrain caspase activity, ensuring proper cellular differentiation and preventing cell death. How caspases are activated in each cellular context remains an open question that should be addressed in the future.

Bcl-2 Family Members

In vertebrates, the Bcl-2 family regulates the mitochondrial pathway of apoptosis by complex interactions that dictate the integrity of the outer mitochondrial membrane, and subsequent release of cytochrome *c* [148]. Bcl-2 family includes over 20 members that are functionally classified as either anti-apoptotic or pro-apoptotic, grouped into three subfamilies based on the number of Bcl-2 homology (BH) domains they share. Most cells express a variety of anti-apoptotic and pro-apoptotic Bcl-2 proteins, and the regulation of their interactions dictates survival or commitment to apoptosis. The anti-apoptotic subfamily of proteins includes Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, and A1/Bfl-1, which possess four BH domains (BH1-4). The next two subfamilies are pro-apoptotic proteins characterized by the presence of three BH (BH1-3) domains, represented by Bax, Bak, and Bok; and BH3-only proteins characterized by the presence of the BH3 domain only, including Bik, Egl-1, Bim, Bmf, Noxa, Bid, Bad, Bnip3, and Beclin-1 [149]. Importantly, BH domains serve as mediators for protein/protein interactions, forming either homo- or heterodimers. Intriguingly, immunohistochemistry of human and mouse embryonic and fetal tissues has disclosed high expression of Bcl-2 in organ systems undergoing morphogenesis and differentiation [150, 151], including neural cells of the developing brain [152, 153].

Bcl-2 Family Members in Neural Differentiation Bcl-2-deficient mice are viable and no severe abnormality in the neuronal system was encountered [154, 155]. In the peripheral nervous system, however, more direct evidence from Bcl-2 knockout mice indicates that Bcl-2 expression is required for the normal progression of a particular early maturational change in embryonic sensory neurons [156]. Furthermore, Bcl-2 proteins are subject to developmental regulation in the nervous system, with high expression levels in neuroepithelial cells of the ventricular zones, and in post-mitotic neurons before adulthood [153]. Bcl-2 immunoreactivity in the brain of squirrel monkeys was shown to be associated with areas involved in neurogenesis and morphogenesis [157]. Therefore, it has been suggested that Bcl-2 might be important, though not crucial, during

neuronal cell differentiation. In vitro, Bcl-2 expression was associated with both neural differentiation of human neural-crest-derived tumor cell line, Paju [158], and retinoic acid-mediated differentiation of CNS neurons [159]. This is in agreement with the observation that the *bcl-2* gene is induced by retinoic acid [160]. In PC12 cells, in turn, Bcl-2 was shown to trigger differentiation as a result of prevention of cell death induced by serum free medium [161]. In the absence of Bcl-2, NGF-mediated differentiation resulted in neurite elongation, whereas Bcl-2 overexpression accelerated neurite formation [162]. On the other hand, Bcl-2 overexpression was reported to increase neurite extension, but differentiation markers were not affected [163]. In fact, Bcl-2 may be a direct effector of neuronal differentiation by influencing MAPK signaling pathways. It has recently been shown that Bcl-2 gene expression is directly correlated with p38α activation and triggers the formation of neuronal networks when overexpressed in a global p38α-repressed environment [164]. Furthermore, specific activation of c-Jun N-terminal kinase (JNK) appears to be involved in Bcl-2-mediated neurite extension of MN9D dopaminergic neuronal cells overexpressing Bcl-2 [163]. Taken together, these data indicate that Bcl-2 may act as an intercellular factor that controls neurite extension and axonal regeneration. Noteworthy, it has been shown that transplantation of ESC overexpressing Bcl-2 further increases the survival of transplanted ESC, as well as neuronal differentiation, and functional outcome [165], indicating that manipulation of Bcl-2 should be valuable for improving transplantation efficacy of NSC.

Bcl-x_L is the most potent anti-apoptotic protein among Bcl-2 family members, both in vitro and in vivo [166, 167]. More specifically, Bcl-x_L is essential for neuronal survival during brain development and in the adult CNS [168, 169]. Similarly to Bcl-2, Bcl-x_L expression increases through the early developmental phase of the brain and peaks during the neurogenesis period [167]. Bcl-x_L was shown to facilitate in vitro differentiation of both mouse and human ESC into the neuronal lineage [170, 171]. Moreover, recent data from conditional Bcl-x_L-mutant mice add support to these observations [172, 173]. Bcl-x_L directs the fate of both immortalized and naïve human NSC and enhances neuron generation by increasing proliferation of neuronal progenitors, while decreasing glia production [174]. These findings support the possibility that the determination of neuronal versus astroglial lineages is actively regulated by balanced levels of anti- and pro-apoptotic Bcl-2 family proteins in neural precursors [172], as illustrated in Fig. 3.

Bcl-x_L instructively directs the fate of embryonic cortical precursors toward neurons [172]. Furthermore, Bcl-x_L overexpression increased differentiation of mouse ESC into midbrain dopaminergic and hindbrain serotonergic neurons compared with wild-type ESC [171].

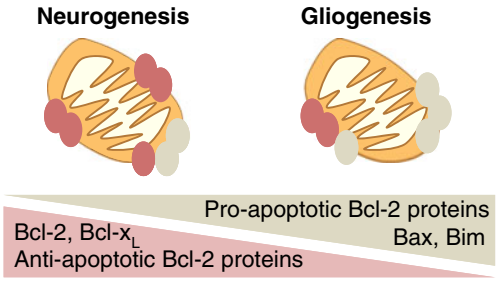

Fig. 3 Control of neural cell specification by Bcl-2 family members. It has been suggested that the determination of neuronal versus astroglial lineages is actively regulated by balanced levels of anti- and pro-apoptotic Bcl-2 family proteins in neural precursors. Both anti-apoptotic members of the Bcl-2 family, *Bcl-2*, and *Bcl-x_L* appear to direct fate of precursor cells toward the neuronal lineage. In contrast, pro-apoptotic members of Bcl-2 family, including *Bax* and *Bim*, repress differentiation into the neuronal phenotype, thus stimulating gliogenesis of neural precursors

Scarce data is available on the role of pro-apoptotic protein Bax during neural differentiation. In fact, during neurodevelopment, Bax expression is coincident with astrocyte formation [175], and declines during postnatal development [167]. In accordance, Bax was shown to instruct embryonic cortical precursors to undergo astrocytic differentiation [172], showing no effect on neuronal differentiation. Bax levels decreased or remained unchanged in differentiating PC12 cells [176] and retinoic acid-mediated differentiation of NTera-2/DI cells [177], respectively.

Finally, the BH3-only protein Bim was recently added to the list of pro-apoptotic proteins with a function in neural differentiation. Using gene-deficiency mice, it has been reported a possible role for the pro-apoptotic protein Bim in neurogenesis of adult-born neural precursor cells [178]. Despite the fact that *bim* and *puma* deficiency does not change early markers of neuronal differentiation in dentate gyrus, bromodeoxyuridine/NeuN double-labeling revealed that the deficiency of *bim*, but not *puma*, accelerates the differentiation of newly generated cells into a neuronal phenotype. In apoptotic context, Bim binds to and "neutralizes" Bcl-2. Therefore, it is possible that endogenous Bim levels determine the amount of sequestered Bcl-2, and by doing so, influence neuronal differentiation or axonal outgrowth.

Finally, the neurogenesis process may also be affected by granzyme B. This serine protease mediates apoptosis by cleaving and activating the pro-apoptotic Bcl-2 family member Bid [179]. Recently, it has been reported that in activated immune T cells, granzyme-B signals potently inhibit neurogenesis, while increasing astroglial differentiation [180]. This study corroborates the contribution of pro-apoptotic Bcl-2 family proteins in determining astroglial phenotype. Nevertheless, the molecular mechanisms underlying the balance of Bcl-2 family members in cell fate decision remains obscure.

Concluding Remarks

Our knowledge of the apoptotic core machinery is immense; however, the continued discovery of new roles for apoptosis-relevant molecules in diverse cellular processes suggests that the majority of what we need to learn is still hidden from us. These studies identify a strong correlation between apoptosis components and regulation of cellular differentiation. Clearly a number of apoptosis executioners and regulators are also important gauges of "stemness" and differentiation of NSC. The mechanistic reach of these molecules extends well-beyond induction of apoptosis and encompasses a multifaceted system of cell fate decision control. Specifically, the complex outcome for components of the apoptosis machinery during neural differentiation suggests their involvement in cell cycle control, cytoskeletal modification, morphogenetic regulation of cell shape, cell migration, and/or switch-like events in several phases of the differentiation process. In addition, PTM of apoptosis regulatory proteins and their specific targets during neuronal differentiation are certainly unique, and different from those in apoptosis context. Future studies will surely reveal these molecules and their interacting partners as possible diagnostic biomarkers of NSC fate and ignite a wave of research to modulate apoptosis regulators toward a more efficient use of stem cells.

Acknowledgments The authors thank all the members of the laboratory for critical reading of the manuscript. This study is supported by grant PTDC/SAU-NMC/117877/2010 from Fundação para a Ciência e a Tecnologia, Lisbon, Portugal.

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