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Review

The emerging multiple roles of nuclear Akt

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ABSTRACT

Akt is a central player in the signal transduction pathways activated in response to many growth factors, hormones, cytokines, and nutrients and is thought to control a myriad of cellular functions including proliferation and survival, autophagy, metabolism, angiogenesis, motility, and exocytosis. Moreover, dysregulated Akt activity is being implicated in the pathogenesis of a growing number of disorders, including cancer. Evidence accumulated over the past 15 years has highlighted the presence of active Akt in the nucleus, where it acts as a fundamental component of key signaling pathways. For example, nuclear Akt counteracts apoptosis through a block of caspase-activated DNase: deoxyribonuclease and inhibition of chromatin condensation, and is also involved in cell cycle progression control, cell differentiation, mRNA: messenger RNA export, DNA repair, and tumorigenesis. In this review, we shall summarize the most relevant findings about nuclear Akt and its functions.

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1. Introduction

Akt is a 56-kDa member of the AGC serine/threonine protein kinase family, discovered in 1991 [1,2]. In mammals, there are three genes, located on different chromosomes, encoding for Akt1/α, Akt2/β, and Akt3/γ. While Akt1 and Akt2 are ubiquitously expressed [3], Akt3 is found predominantly in brain, kidney, and heart [4]. Akt isoforms share a high degree of sequence homology in their catalytic domains, but diverge in other regions of the protein, i.e. the C-terminus regulatory domain and the N-terminus pleckstrin homology (PH) domain [3] (Fig. 1). The kinase catalytic domain, located in the central region of the protein, displays a high degree of similarity to those found in protein kinase A (PKA) and protein kinase C (PKC), hence the other name of Akt, protein kinase B (PKB) [5].

All three Akt isoforms undergo membrane recruitment through the N-terminus PH domain which binds to phosphatidylinositol 3,4,5-trisphosphate (PIP3) synthesized by phosphatidylinositol 3-kinase (PI3K), leading to phosphorylation and activation of Akt kinase activity [6]. The three isoforms contain similar phosphorylation sites: Thr 308 (Akt1)/Thr 309 (Akt2)/Thr 305 (Akt3) and Ser 473 (Akt1)/Ser 474 (Akt2)/Ser 472 (Akt3) [3] (Fig. 1). The Thr residues are phosphorylated by phosphoinositide-dependent protein kinase 1 (PDK1), which is also recruited to the plasma membrane by PIP3 [7,8]. In contrast, the Ser residues are targeted by mammalian target of rapamycin complex 2 (mTORC2) [9]. However, the Ser residues can be phosphorylated by other kinases, including integrin-linked kinase (ILK) [10]. Together, the two phosphorylation events cause full activation of Akt, however, in the absence of Ser 473 phosphorylation, Thr 308 p-Akt can still phosphorylate some, but not all, of its substrates [11].

Akt is inactivated through the action of protein phosphatases. The Thr residues are dephosphorylated by protein phosphatase 2A (PP2A) [12]. The core enzyme of PP2A is a dimer consisting of a catalytic subunit (PP2A/Cα or β) and a regulatory/structural A subunit (PP2A/Aα or β). A third regulatory B subunit (PP2A/B), that determines substrate specificity, can be associated with this core structure [13].

The Ser residues are targeted by the pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) family of isozymes. Three PHLPP isoforms have been identified so far, the alternatively spliced PHLPP1α and PHLPP1β, and PHLPP2 [14]. These PHLPP isoforms are involved in the dephosphorylation of specific Akt isozymes. PHLPP1 targets Akt2 and Akt3, while PHLPP2 dephosphorylates Akt1 and Akt3 [15].

Akt was first characterized for its function in regulating cell proliferation and survival. This could be due to the direct or indirect effects

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of Akt on many cell proteins. Accordingly, constitutive activation of Akt leads to cell cycle dysregulation and inhibition of pro-apoptotic pathways that are typical hallmarks of human tumors [16]. Therefore, over-activation of Akt signaling is oncogenetic [17]. Consistently, Akt activity is up-regulated in many types of neoplasia where it is involved in drug-resistance [18]. For this reason, Akt is regarded as a potential therapeutic target for innovative treatments of cancer, and some Akt inhibitors are now being tested in clinical trials in cancer patients [19,20].

Once activated, Akt isoforms translocate to various subcellular compartments, including the endoplasmic reticulum, the mitochondria, the Golgi, and the nucleus, where they phosphorylate substrates or interact with other cell components.

Here, we shall highlight the existing evidence on the multiple roles played by Akt in the nucleus. However, for a better understanding of this review, it is useful to briefly recapitulate some general data regarding both Akt and nuclear structure.

2. Functions and substrates of Akt

Akt constitutes an important node in many signaling cascades downstream of growth factor receptor tyrosine kinases and G protein-coupled receptors. As such, Akt isoforms play key roles in cell survival [17], proliferation [21], growth [22], migration [23], polarity [24], insulin-evoked glucose transport [25], glucose and lipid metabolism [26,27], contractility in both skeletal muscle [28] and cardiomyocytes [29], angiogenesis [30], and self-renewal of stem cells [31–33]. Impaired Akt activity has been associated not only with cancer [34–36], but also with other disorders, that include type 2 diabetes, cardiovascular and neurodegenerative diseases, and muscle hypotrophy [37,38].

Akt substrates often contain the phospho-Akt-substrate consensus motif (R-X-R-X-X-S/T) [39]. Consensus motif analysis indicates that there are potentially thousands of cellular substrates for Akt, however only about 60–70 of these have been characterized so far. Akt may either positively or negatively affect the functions of these substrates (although many of them are usually inhibited), alter their subcellular localization, or modify their stability.

Promoters of apoptosis that are inhibited by Akt include the FOXO family members of transcription factors, FOXO1a, FOXO3a, and FOXO4 [40,41]. Phosphorylation of FOXO family members by Akt leads to their nuclear exclusion and inactivation, with a consequent decreased transcription of mRNAs coding for proteins (BH3-only family proteins and Fas-ligand, for example) that are required for promoting apoptosis [42,43]. However, Akt delivers anti-apoptotic signals through proteins whose function is directly modulated by Akt phosphorylation. Bad was one of the first discovered targets of Akt [44]. Bad is a pro-apoptotic member of the Bcl-2 family of proteins, which binds Bcl-2 or Bcl-XL, thus blocking their anti-apoptotic activities. Phosphorylation of Bad on Ser 136 by Akt disrupts Bad interactions with Bcl-2/Bcl-XL, localized on the outer mitochondrial membrane. As a consequence, Bad is sequestered in the cytosol, through the interaction with 14-3-3 protein [45]. In an analogous way, phosphorylation by Akt of pro-apoptotic Bax on Ser 184 blocks Bax translocation to mitochondria, thus preventing Bax conformational change, an event that occurs after apoptotic induction [46]. Akt phosphorylates apoptosis signaling kinase 1 (ASK-1) at Ser 83, which attenuates ASK-1 activity and promotes cell survival, as ASK-1 transduces stress signals to the pro-apoptotic jun NH2-terminal kinase or JNK and p38 MAP kinase cascades [47].

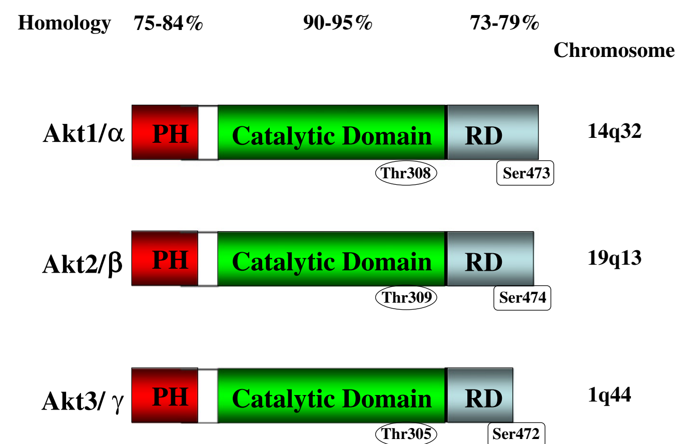

Fig. 1. Domain structure of Akt isoforms. All the Akt isoforms possess a catalytic (kinase) domain in the central region of the molecule. The PH domain acts as a phosphatidylinositol 3,4,5-trisphosphate-binding module. The RD is located at the C-terminus, adjacent to the kinase domain. Phosphorylation sites in the activation loop of the catalytic domain and in the RD are indicated. The percentage of sequence homology of the three isoforms as well as the chromosome location of the genes coding for the Akt isozymes are also highlighted. Abbreviations: PH, pleckstrin homology; RD, regulatory domain.

Targets activated by Akt for promoting cell survival, include the inhibitor of kappa B kinase-α (IKKα), which in turn phosphorylates and thereby promotes the degradation of the inhibitory cofactor of NF-κB, I-κB [48]. This allows NF-κB to translocate to the nucleus where it stimulates the transcription of pro-survival genes, such as those coding for inhibitor of apoptosis protein (IAP) 1 and IAP2 [49].

Akt positively regulates cyclic AMP-response element binding protein (CREB) activity, however in this case by direct phosphorylation [50,51]. CREB phosphorylation at Ser 133 by Akt then induces the binding of accessory proteins that are necessary for the transcription of anti-apoptotic genes, including Bcl-2 and Mcl-1 [49,52]. Murine double minute 2 (MDM2) is an E3 ubiquitin ligase whose expression is induced by the p53 tumor suppressor. As a feed-back loop, MDM2 negatively controls intracellular levels of p53, a major cell death effector [53]. Phosphorylation of MDM2 at Ser 166 and Ser 186 by Akt decreases MDM2 self-ubiquitination and renders the protein more stable. As a consequence, p53-mediated apoptosis is inhibited [54].

As to cell proliferation, Akt phosphorylates $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, two members of the Cip/Kip family of cyclin-dependent kinase (CDK) inhibitors. Both $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ associate with CDK/cyclin complexes, and serve to inhibit kinase activity and block progression through the G_1/S transition of the cell cycle [55]. Akt phosphorylates $p21^{Waf1/Cip1}$ at both Thr 145 and Ser 146. Thr 145 phosphorylation results in the cytoplasmic localization of $p21^{Waf1/Cip1}$ and thus promotes the cell cycle [56], whereas the Ser 146 site phosphorylation enhances the stability of the protein and further increases the assembly of cyclin D-CDK4 G_1/S transition complex [43]. Akt-mediated phosphorylation of $p27^{Kip}$ at Thr 157 causes the relocation of $p27^{Kip}$ to the cytoplasm, thus relieving the nuclear substrates (CDK2/cyclin E and CDK2/cyclin A) from $p27^{Kip}$ inhibition and enhancing cell cycle progression [57].

Moreover, Akt phosphorylates and inactivates glycogen synthase kinase (GSK) 3β. GSK3β phosphorylates cyclin D1 at Thr 286 [58] and Myc at Thr 58 [59], which promotes their nuclear export and degradation via the ubiquitin-mediated pathway. Thus, inhibition of GSK3β activity by Akt-mediated phosphorylation facilitates the G_1/S progression of the cell cycle.

Akt plays a key role in promoting cell growth. The predominant mechanism appears to be through the activation of the mammalian target of rapamycin complex 1 (mTORC1), which is regulated by both nutrients and growth factor signaling [60]. Akt phosphorylates 200-kDa tuberous sclerosis 2 (TSC2 or hamartin). TSC2 is a GTPase activating protein (or GAP) protein which associates with 130-kDa tuberous sclerosis 1 (TSC1 or tuberin) to inactivate the small G protein Ras homolog enriched in brain (Rheb). Once phosphorylated by Akt at Thr 1462 [61], TSC2 binds 14-3-3 proteins [62]. Upon binding to 14-3-3 proteins, TSC2 is degraded by the proteasome, and Rheb accumulates in a GTP-bound state, which activates mTORC1 [63]. Moreover, another Akt substrate is involved in mTORC1 control, the

proline-rich Akt substrate of 40 kDa (PRAS40), which acts as a negative regulator of mTORC1 activity [64]. Akt directly phosphorylates PRAS40 on Thr 246 [65], and this phosphorylation is fundamental for 14-3-3 protein binding to PRAS40 which relieves mTORC1 from PRAS40 inhibition [66].

Nevertheless, it should be emphasized that Akt regulation of mTORC1 is important for cell proliferation and survival, as mTORC1 controls the translation of numerous proteins involved in cell cycle progression and anti-apoptotic signaling [67].

3. Nuclear structure

The nucleus is the organelle where processes such as DNA replication, transcription, and mRNA processing take place. Major advances in cytological methods and molecular genomics have provided fundamental insights on nuclear organization. The nucleus is separated from the cytoplasm by the nuclear envelope (NE) that compartmentalizes the genetic material from the protein translation machinery in the cytoplasm. The NE is a highly organized membranous structure comprising the outer and inner nuclear membranes, nuclear pore complexes (NPCs), and the nuclear lamina [68]. The outer nuclear membrane is directly continuous with the rough endoplasmic reticulum containing ribosomes while the inner nuclear membrane contains unique sets of intrinsic and extrinsic proteins that bind the nuclear lamina and chromatin [69]. NPCs serve as aqueous channels for nuclear import/export of high molecular weight molecules [70].

The nuclear lamina is an intermediate filament protein meshwork anchored to the inner nuclear membrane which provides structural support to the nucleus and interacts directly with chromatin [71,72]. As to the nuclear interior, several lines of evidence suggest that it displays a compartmentalized structure, consisting of chromosome territories (CTs) and an interchromatin compartment (IC). CTs comprise a hierarchy of chromatin domains starting with DNA loop domains with an average DNA content ranging from about 30 to 200 kilobases (kb), referred to as 100 kb chromatin domains. This model of nuclear organization predicts that a series of loop domains forms larger chromatin domains with DNA contents of several hundred kb to several megabases (Mb), referred to as 1 Mb chromatin domains [73]. Large chromosomes are found more often at the nuclear periphery, while small chromosomes are found more interiorly. However, the spatial separation of chromosomes in the nucleus is not absolute, as chromosomal intermingling occurs at the periphery of the territories. A further division can be found within the CTs, with gene-poor and gene-rich regions spatially separated [74]. Gene-poor CTs and silenced genes are frequently found in association with the nuclear periphery, a similar location to that of heterochromatin. In contrast, gene-rich CTs and active genes localize to the nuclear interior [75]. The IC has been envisaged as a three-dimensional network of lacunas and channels, which starts at the nuclear pores and then expands both between neighboring CTs and into the interior of individual CTs. The IC is lined by the surface of smaller and larger chromatin domains [76]. It has been suggested that the IC and the border zone between chromatin domains and the IC, referred to as the perichromatin region, possess a defined topology that is essential for transcription, RNA splicing, DNA replication, and presumably also for DNA repair [77].

It should be considered that the nucleus is unique amongst cellular organelles as it contains a myriad of discrete suborganelles, referred to as nuclear bodies [78]. Nuclear bodies are morphologically and molecularly distinct dynamic entities that further compartmentalize the IC. Therefore, they create microenvironments within the nucleus where specific nuclear processes take place. At variance with cytoplasmic organelles, nuclear bodies are not surrounded by lipid membranes, and their structural integrity is completely mediated by protein–protein and, possibly, by protein–RNA interactions [78].

4. Nuclear Akt

The presence of active, phosphorylated Akt (p-Akt) within the nucleus has been reported since the late 1990s. Indeed, some of Akt substrates are resident in the nucleus, such as the FOXO family of transcription factors [79] or the transcriptional coactivator p300 [80]. Akt1, Akt2, and Akt3 have been reported to reside in the nucleus or to migrate into the nucleus in response to a wide variety of stimuli that include insulin-like growth factor-1 (IGF-1), F(ab′)2 fragment of anti-mouse IgG acting on B-cell receptor, hypoglycemia, insulin, and nerve growth factor (NGF) [81–86].

The nuclear localization sequence (NLS) motif of Akt is unknown, however the proto-oncogene T-cell leukemia-1 (TCL1) protein family may be involved in Akt nuclear localization, as first demonstrated in human T-cell leukemia [87]. Three TCL1 isoforms have been identified in both the human and the mouse genome: TCL1, TCL1B, and MTCP1 (mature T cell proliferation 1) [88]. TCL1 binds to the PH domain of Akt and mediates the formation, at the plasma membrane level, of TCL1-Akt high-molecular-weight protein complexes. Akt is then preferentially phosphorylated and activated within these complexes [89]. In two-cell mouse embryos that were genetically deficient in TCL1, phosphorylated Akt had a striking cortical localization and was lacking in blastomere nuclei [90]. Moreover, when the levels of TCL1B were reduced by siRNA in T47-D breast cancer cells, a decrease in the levels of nuclear (but not cytoplasmic) p-Akt was observed. Conversely, when TCL1B was overexpressed in either MCF-7 or T47-D cells, an increase in the amount of nuclear p-Akt was detected [91]. These findings highlighted the important role played by TCL1 in nuclear targeting of Akt. However, further studies are necessary to establish whether the TCL1–Akt interactions are a universal mechanism required for nuclear import of Akt. Indeed, while TCL1 proteins are usually expressed at high levels in embryonic/fetal cells and in various stages of B- and T-cell development, their levels in healthy adult tissues are low, at least in mouse [88]. Nevertheless, the expression of TCL1 proteins is up-regulated in some solid and hematologic cancer types [91,92]. This finding could somehow explain the increased nuclear localization of Akt which is observed in some kinds of cancer.

Whether or not Akt needs to be phosphorylated for entering the nucleus is controversial. Indeed, it has been documented that unphosphorylated Akt could migrate to the nucleus in HEK293 cells overexpressing kinase-dead Akt mutants (T308A, S473A) that could not be phosphorylated, suggesting that phosphorylation of Akt was not required for its nuclear localization [93]. In contrast, overexpression of the same mutants impaired Akt nuclear translocation after NGF stimulation of PC12 cell [94]. It was therefore concluded that activity and phosphorylation of Akt kinase were involved, at least to some degree, in Akt nuclear translocation [94]. It is conceivable that these discrepancies are dependent on the different cell types/stimuli used. Another controversial issue regards whether or not Akt can be phosphorylated within the nucleus. The nucleus contains all the machinery necessary for phosphorylating Akt at Thr 308, including PI3K [95], PIP3 [96], and PDK1 [97,98]. Moreover, mTORC2 has been demonstrated to localize to the nucleus in aggressive variants of papillary thyroid carcinomas, concomitantly with high levels of Ser 473 p-Akt. Therefore, it has been hypothesized that mTORC2 could phosphorylate Akt at Ser 473 within the nucleus of thyroid neoplastic cells [99]. In this connection, it is interesting that mTORC2 has been localized to both the cytoplasm and the nucleus, whereas mTORC1 is predominantly cytoplasmic [100], even if an mTORC1 component, Raptor, has been recently identified as a phosphoprotein involved in rDNA transcription in nucleoli [101,102].

Nevertheless, several lines of evidence indicate that Akt migrates to the nucleus after having been phosphorylated at the plasma membrane [103,104] and that nuclear PDK1 is not involved in phosphorylating Akt at Thr 308. Rather, it seems that PDK1 nuclear translocation

may be a mechanism to sequestrate it from activation of cytosolic signaling pathways [105]. However, other investigators have come to opposite conclusions [106].

All three Akt isoforms possess a classic, leucine rich, leptomycin-sensitive nuclear export sequence (NES). Accordingly, stable over-expression of Akt1 displaying a non-functional NES, resulted in persistent nuclear localization of Akt1 and enhanced cell migration in vitro of Akt1$^{-/-}$ fibroblasts [93].

5. Functions of nuclear Akt

5.1. Cell cycle progression

Considering the paramount role played by Akt in cell cycle regulation, it is not surprising that Akt translocated to the nucleus during early G_1 phase of the cell cycle [107]. Moreover, MCF7 breast cancer cells overexpressing a ΔNES-mutant (L277A/L280A/L282A) Akt1, contained higher levels of nuclear p-Akt compared with cells overexpressing the empty vector only. Higher levels of nuclear Akt correlated with increased cyclin D1 expression and cell proliferation in response to estradiol challenging [91]. Increased expression of cyclin D1 by nuclear Akt may be related to the fact that Akt up-regulates the histone acetyltransferase activity and transcriptional activity of p300 [80] which is involved in the histone acetylation at the cyclin D1 promoter and cyclin D1 gene transcription [108].

When a rapid depletion of nuclear p-Akt was induced by extracellular ATP or statins in insulin-stimulated A549 cells, PHLPP1 and PHLPP2 were recruited to the nucleus. PHLPP1 and PHLPP2 were instrumental for Akt dephosphorylation, which, however, required nuclear translocation of the PIP3 phosphatase, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [109]. PTEN is a tumor suppressor, as it down-regulates oncogenic PI3K/Akt signaling by dephosphorylating PIP3 [110]. Upon nuclear Akt dephosphorylation and depletion, proliferating cell nuclear antigen (PCNA) and p21$^{Waf1/Cip1}$ translocated to the nucleus where they formed a complex. It is known that nuclear PCNA/p21$^{Waf1/Cip1}$ complexes cause cell cycle arrest [111,112]. p110β PI3K was essential for nuclear export of p-Akt in insulin-stimulated cells exposed to either statins or elevated levels of extracellular ATP [113]. These findings are consistent with a report which has documented an association between p110β PI3K and Akt at the nuclear level [114]. Nuclear p110β PI3K is involved in DNA replication, as its catalytic activity was required for regulating the nuclear activation of Akt during the S phase of the cell cycle and in turn the phosphorylation of the cell cycle progression inhibitor, p21$^{Waf1/Cip1}$ [114].

Overall, these findings have highlighted some signaling events through which nuclear Akt could control cell cycle progression.

5.2. Cell survival

Akt signaling blocks cell death both by impinging on the cytoplasmic apoptotic machinery and by mediating the expression of genes involved in cell death and survival. However, there is strong evidence documenting a more direct role for nuclear Akt in apoptosis suppression. This function of Akt has been mainly investigated in PC12 neural cells challenged with NGF and primary cardiomyocytes.

5.2.1. PC12 cells

NGF is the prototypical member of a family of neurotrophic factors called neurotrophins that play key roles in cell survival, differentiation, and growth arrest [115,116]. NGF stimulated phosphorylated Akt to translocate to the nucleus of PC12 cells [86]. In a series of seminal papers, Ye and coworkers have investigated the molecular mechanisms underlying the anti-apoptotic effects of nuclear Akt in PC12 cells challenged with NGF. They overexpressed wild-type, constitutively active or dominant-negative Akt in PC12 cells, and treated the cells with NGF. Upon NGF treatment, the nuclei isolated from control cells, or from cells overexpressing either wild-type or constitutively active Akt, were resistant in vitro to internucleosomal cleavage caused by a pro-apoptotic apoptotic solution, consisting of HEK293 cell cytosol supplemented with purified active caspase-3 [117]. In contrast, they observed DNA fragmentation in dominant-negative Akt samples in spite of NGF treatment, indicating that nuclear Akt was required for NGF-mediated anti-apoptotic signaling. Nevertheless, without NGF treatment, all the nuclei displayed DNA fragmentation even if they had been isolated from cells overexpressing constitutively active Akt. This indicated that Akt activation alone was not sufficient to inhibit DNA cleavage [118]. In an attempt to search for nuclear Akt substrates contributing to blocking apoptosis, the same group identified acinus, a nuclear factor required for apoptotic chromatin condensation after cleavage by caspase-3 [119]. Acinus is cleaved by caspase-3 on both its N- and C-termini, producing a p17 active form, which triggers chromatin condensation even in the absence of caspase-3. Full-length acinus is unable to induce chromatin condensation, suggesting that caspase-mediated cleavage is necessary for this activity [120]. Acinus, which resides in nuclear speckles (a type of nuclear bodies), is a direct target of Akt [121]. Akt phosphorylation of acinus on both Ser 422 and Ser 573 resulted in acinus resistance to caspase cleavage in the nucleus and inhibition of acinus-dependent chromatin condensation. If acinus phosphorylation by Akt was abolished through mutagenesis, its proteolytic degradation and chromatin condensation were accelerated. Moreover, overexpression of an acinus mutant that mimicked phosphorylation by Akt and was resistant to apoptotic cleavage, prevented chromatin condensation [121].

Intriguingly, upon NGF stimulation of PC12 cells, nuclear Akt phosphorylated zyxin, an 82-kDa focal adhesion protein that shuttles between the nucleus and the cytoplasm and that antagonizes apoptosis [122]. Upon phosphorylation at Ser 142 by Akt, nuclear zyxin robustly interacted with acinus and suppressed its apoptotic cleavage, resulting in inhibition of apoptotic chromatin condensation [123]. Therefore, nuclear Akt provides a dual protection on acinus by inducing a direct binding between zyxin and acinus and blocking its proteolytic degradation, in addition to direct phosphorylation of acinus.

In some cases, nuclear Akt is involved in NGF-dependent cell survival through interactions with other proteins, that, however, are not Akt substrates. One such protein is represented by ErbB-3 binding protein (Ebp1), a ubiquitously expressed protein, which localizes in both the nucleus and the cytoplasm and binds ErbB3 receptor in human serum-starved breast cancer cell lines [124]. There are two Ebp1 isoforms, p48 and p42. The longer-form p48 localizes in both the cytoplasm and the nucleus, whereas the shorter-form p42 predominantly resides in the cytoplasm [125]. The p48 Ebp1 isoform was identified in nuclear extracts prepared from NGF-challenged PC12 cells as a protein which contributed to inhibition of DNA fragmentation by caspase-activated DNase (CAD), also referred to as DNA fragmentation factor or DFF. Nuclear (but not cytoplasmic) p-Akt associated with PKC-phosphorylated Ebp1 and enhanced its anti-apoptotic action. However, the kinase activity of Akt was not required for Ebp1 binding. Ebp1 interacted with the N-terminus PH and catalytic domains of Akt [126]. PKC-δ has been subsequently identified as the PKC isoform that phosphorylates Ebp1 at Ser 360, an event which prevented caspase-3-mediated proteolytic cleavage and that was necessary for Ebp1/Akt interactions [127].

Another nuclear Akt-interacting protein is B23, also referred to as nucleophosmin. B23 mainly localizes to the nucleolus and is thought to play key roles in diverse cellular functions that include ribosome biogenesis, cell cycle progression, DNA repair, centrosome duplication, and response to stress [128]. Furthermore, it is involved in the pathogenesis of several human cancers, including acute myelogenous leukemia (AML) [129]. Intriguingly, it has been described both as an activating oncogene and a tumor suppressor, depending on cell type

and protein levels. The C-terminus of B23 strongly interacted with the PH domain of Akt. Akt binding to B23 in response to NGF stimulation of PC12 cells, protected B23 from proteolytic degradation by caspase-3 in vitro and in vivo, leading to the up-regulation of cell survival. Nuclear Akt phosphorylation, but not its kinase activity, was required for Akt association with B23, in analogy with Akt interactions with Ebp1 [130]. Moreover, a nuclear B23/Akt/PIP3 complex inhibited DNA fragmentation effected by CAD/DFF, which is the major apoptotic nuclease [131,132]. Overall, the above-highlighted findings presented convincing evidence that nuclear Akt inhibits apoptosis not only through phosphorylation of transcription factors (e.g. FOXO proteins) but also through the direct targeting of apoptotic effectors operating in the nucleus. Anti-apoptotic signaling controlled by Akt in PC12 cells treated with NGF is highlighted in Fig. 2.

5.2.2. Cardiomyocytes

The anti-apoptotic role of nuclear Akt has been studied in some detail in cardiomyocytes. Apoptosis occurs in a wide variety of cardiovascular disorders and is now recognized as a fundamental process that contributes to deterioration of cardiac function characterizing heart failure [133]. The key pathophysiological process that ultimately leads to chronic heart failure is cardiac remodeling (mainly due to cardiomyocyte hypertrophy) in response to chronic disease stresses, such as, for example, pressure overload due to hypertension [134]. It is well established that short-term Akt activation promotes physiological heart hypertrophy and protection from myocardial injury, whereas, long-term Akt up-regulation causes pathological hypertrophy and heart failure [29]. Interestingly, it seems that if p-Akt resides in the nucleus, the negative effects of its up-regulated activity may be avoided [135]. Akt was selectively targeted to the nucleus of neonatal rat ventricular cardiomyocytes by using an adenovirus shuttle vector in which full-length wild-type mouse Akt cDNA was cloned upstream of three in-frame NLS sequences. Akt targeting to the nucleus of cardiomyocytes did not result in evidence of morphological remodeling, such as altered myofibril density or hypertrophy. Nuclear extracts from transfected cardiomyocytes displayed elevated levels of both Thr 308 and Ser 473 p-Akt, as well an increase in Akt kinase activity. In contrast, the levels of cytoplasmic Ser 473 p-Akt were similar to control animals. Moreover, nuclear targeted Akt was as effective as myristoylated-Akt in blocking staurosporine-, deoxyglucose-, and hypoxia-induced apoptosis of cardiomyocytes [136]. Also, cardiomyocytes from transgenic mice were protected against ischemia–reperfusion injury and did not display increased levels of phosphorylated Bad and GSK-3β, two cytoplasmic Akt substrates. Of note, GSK-3β is involved in cardiomyocyte hypertrophy [137].

Taken together, these findings suggested that nuclear targeting of Akt prevented apoptosis without inducing hypertrophic remodeling, thus opening new possibilities for therapeutic applications to inhibit cell death associated with heart disorders. Accordingly, Ser 473 p-Akt levels increased in the nucleus of mouse cardiomyocytes after infarction challenge. Therefore, Akt translocation to the nucleus could be interpreted as a physiological mechanism aiming to protect cells from death due to oxygen deprivation [106].

A subsequent study from the same group documented that nuclear overexpression of Akt in mice did not modify left ventricular free wall thickness, chamber diameter, longitudinal axis, and volume, but rather resulted in an increased number of cardiomyocytes, that, however, were smaller in volume [138]. Moreover, the heart from transgenic animals was characterized by enhanced performance involving both systolic and diastolic function, that were paralleled by increased performance in the contraction of isolated ventricular cardiomyocytes. Indeed, cardiomyocytes' shortening and velocity of shortening/relengthening were increased and coupled with a more efficient reuptake of Ca^{2+} by the sarcoplasmic reticulum (SR). The enhanced SR function was dependent upon an increase in SR Ca^{2+}-ATPase2a activity sustained by higher levels of phospholamban (PLN) phosphorylation. PLN regulates the function of sarcoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a, the predominant contributor of the Ca^{2+} influx into the SR), and the unphosphorylated form of PLN inhibits SERCA2a and thereby the reuptake of Ca^{2+} by the SR. In contrast, the phosphorylated form of PLN enhances the activity of SERCA2a and the transport of Ca^{2+} into the SR [139]. PLN hyperphosphorylation was associated with an increase in p-PKAα and a decrease in protein phosphatase-1 (PP1) levels. Taken together, these observations provided a likely biochemical mechanism for the potentiation of cardiomyocyte and ventricular function in nuclear Akt transgenic mice, however it remains to be established how increased expression of Akt in the nucleus could impact on p-PKAα and PP1 levels [138].

It is very interesting that young women displayed higher levels of nuclear-localized Ser 473 p-Akt relative to comparably aged men or postmenopausal women. Considering that cardiovascular disease risk is higher in men or postmenopausal women than in young women, it is tempting to speculate that the activation of Akt in the nucleus in a gender- and age-dependent manner may help to somehow explain the differences observed in cardiovascular disease risk [140]. Both the localization of Ser 473 p-Akt in cardiomyocyte nuclei of sexually mature female mice versus males and Akt kinase activity in nuclear extracts of hearts from female mice versus males were elevated [140]. The differences could be dependent on estrogen, as Ser 473 p-Akt was also localized to the nucleus of cultured mice cardiomyocytes after exposure to 17β-estradiol or genistein (a phytoestrogen present in soy protein-based diets), and neonatal exposure of mouse litters to genistein increased nuclear localization of Ser 473 p-Akt [140]. Therefore, increased levels of active Akt in the nucleus could somehow explain the protective effects of estrogens against heart disorders [141].

Atrial natriuretic peptide (ANP) is a physiological regulator of fluid retention and blood pressure which could be of clinical benefit in some cardiovascular disorders such as hypertension, myocardial infarction, coronary heart disease, and cardiac failure [142]. Overexpression of Akt in the nucleus induced ANP gene expression in vitro in murine cardiomyocytes in a pressure overload model [135]. Moreover, exogenous ANP promoted nuclear accumulation of Ser 473 p-Akt and protected cardiomyocytes from staurosporine-mediated apoptosis. Intriguingly, nuclear targeting of Akt in cardiomyocytes was accompanied by migration to the nucleus of zyxin. Other cardioprotective agents (IGF-1, adrenomedullin, and estrogens) promoted translocation of zyxin to the nucleus [143]. This observation is consistent with the results reported for PC12 cells challenged with NGF [123]. Therefore, both Akt and zyxin could be part of a nuclear signaling network activated by different anti-apoptotic effectors.

5.3. DNA repair

Akt is activated in response to DNA damaging agents such as ionizing radiation (IR) [144]. Activated Akt enhances the DNA damage-induced transcription and promotes cell survival [145]. IR produces DNA double strand breaks (DSB) [146]. Interestingly, DNA-dependent protein kinase (DNA-PK), which is involved in DSB repair [147], has been identified as a putative Ser 473 Akt kinase [148]. Another central player in DSB repair is the ataxia-telangiectasia-mutated (ATM) kinase [149]. It has been shown that a subset of Ser 473 p-Akt accumulated in the nucleus at IR-induced foci where it co-localized with the DBS markers, γ-H2AX and Ser 1981 p-ATM [150]. Ser p-473 Akt induction was independent of both PI3K and DNA-PK, but was downstream of meiotic recombination 11 (MRE11)-dependent ATM activation and ring finger protein 168 (RNF168)-dependent histone ubiquitinylation. MRE11, together with RAD50 and Nijmegen breakage syndrome 1 (or NBS1, also known as nibrin), form the MRE11 complex, a sensor of DSB that controls the DNA damage response by governing the activation of ATM [151]. RNF168 is an E3 ubiquitin ligase which is involved in histone

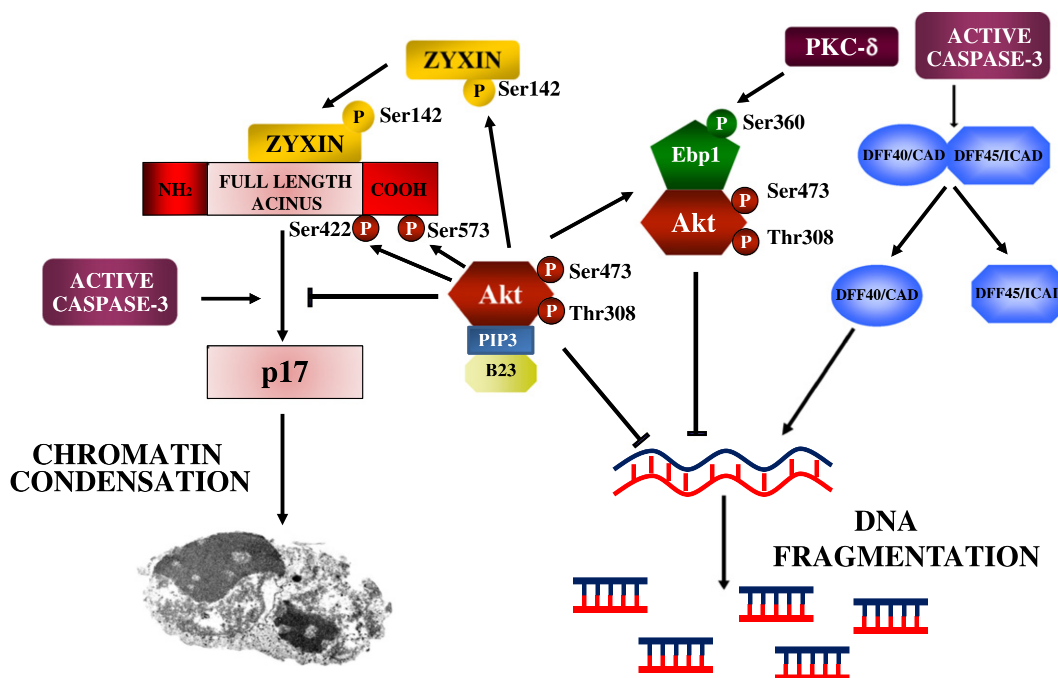

Fig. 2. Schematic of intranuclear anti-apoptotic signaling regulated by active Akt in NGF-challenged PC12 cells. Active (phosphorylated) Akt phosphorylates full length acinus at Ser 422 and Ser 573 and, by doing so, prevents acinus cleavage by caspase-3. This results in the blockage of chromatin condensation. Moreover, Akt phosphorylates zyxin at Ser 142. Phosphorylated zyxin associates with acinus and also this event stops acinus cleavage by caspase-3. In healthy cells, DFF (or CAD) exists in the nucleus as a heterodimer, composed of a 45-kDa chaperone and inhibitor subunit (DFF45/ICAD) and a 40-kDa latent nuclease subunit (DFF40/CAD). Apoptotic activation of caspase-3 results in the cleavage of DFF45/ICAD and release of active DFF40/CAD nuclease that cleaves DNA at the internucleosomal level. Active Akt, through its interaction with B23 and PIP3, blocks DFF40/CAD-dependent DNA fragmentation. To this end, nuclear p-Akt is necessary but not sufficient. Active Akt also associates with Ebp1 phosphorylated at Ser 360 by PKC-δ. The Akt/Ebp1 complex opposes DNA fragmentation by DFF 40/CAD. Abbreviations: CAD, caspase-activated DNase; DFF, DNA fragmentation factor; Ebp1, ErbB-3 binding protein; ICAD, inhibitor of CAD; PKC, protein kinase C; PIP3, phosphatidylinositol 3,4,5-trisphosphate.

H2A and γ-H2AX non-proteolytic poly-ubiquitinylation of the DSB-flanking chromatin, an essential step for restoration of genome integrity [152]. Therefore, these findings linked nuclear p-Akt to DSB repair and cell survival. However, it not clear yet the protein kinase which could phosphorylate Akt at the DSB foci, as ATM is not capable of directly phosphorylating Akt [153].

5.4. RNA export

Many classes of RNA are exported from the nucleus, including tRNA, rRNA, snRNA, and mRNA. A key player in mRNA nuclear export is the protein referred to as REF/Aly, which is recruited to mRNA during splicing [154]. Microinjection of antibodies to Aly blocked mRNA export without affecting other transport mechanisms [155]. Aly localized to nuclear speckles where it interacted with PIP3 [156]. Nuclear Akt phosphorylated Aly on Thr 219 and this phosphorylation was necessary for Aly binding to PIP3. Depletion of Aly by siRNA resulted in reduced cell proliferation and mRNA export, and these two processes required both Aly phosphorylation by Akt and Aly interaction with PIP3 [156]. Thus, Aly phosphorylation could be yet another mechanism by which nuclear Akt controls cell progression, most likely by facilitating the export of mRNAs coding for proteins that are involved in cell cycle regulation.

5.5. Cell differentiation

Akt signaling plays a major role in insulin-dependent adipogenic differentiation [157]. An innovative strategy has been recently developed for the evaluation of the signaling roles played by Akt localized at different subcellular locations during adipogenic differentiation. Selective Akt inhibitor peptides were generated using variations of the Akt substrate recognition sequence (R-X-R-X-X-S/T) which contained alanines in place of the target serines or threonines, cloned in-frame with the cDNA encoding GST. A myc epitope tag was also included, for facilitating peptide immunodetection [158]. Moreover, the inhibitory peptides were engineered in-frame with peptides directing to specific subcellular locations. Thus, for obtaining a selective inhibitor of nuclear Akt, the SV40 large T antigen amino acids 126–132 were used. Selective inhibition of nuclear Akt, affected the localization and the transcriptional activity of FOXO3a in 3T3L1 preadipocytes induced to differentiate into adipocytes through stimulation with insulin and dexamethasone. This resulted in a block of adipogenic differentiation, as documented by analysis of Oil Red O accumulation [158].

Stem cell therapies have been considered as novel strategies for treating heart disorders [159]. However, only relatively modest improvements in cardiac function and structure have been reported so far, at least in part due to insufficient stem cell proliferation and viability after delivery [160]. To improve the outcome of stem cell therapy, it is of critical importance to unravel mechanisms promoting survival and proliferation of transplanted stem cells without inhibiting lineage commitment. Pim-1 is a pro-survival and -proliferation kinase, located down-stream of Akt, which was capable of enhancing myocardial regeneration after infarction [161]. However, targeting of Akt to the nucleus using a cDNA with three in-frame NLS sequences, inhibited the commitment of murine cardiac progenitor cells, and increased their proliferation rate most likely due to enhanced transcription of cell cycle-related genes (cyclin D1, cell division cycle 2 or CDC2, checkpoint kinase 1 or Chk1). Moreover, elevated levels of Ser 133 p-CREB were detected in these genetically engineered cells [162]. These results

were similar to those obtained when nuclear Akt was overexpressed in cardiomyocytes (see above). However, infarcted hearts receiving intramyocardial injection of nuclear Akt-overexpressing progenitor cells did not display long-term functional and structural improvements when compared with controls. Therefore, these findings demonstrated that full lineage commitment of cardiac progenitor cells is essential to the regenerative response in infarcted hearts and that over-expression of nuclear Akt could not be considered as a strategy for improving the outcome of stem cell transplantation in the treatment of myocardial infarction [162].

5.6. Tumorigenesis

The presence of nuclear p-Akt has been reported in lung, breast, prostate, and thyroid cancers, as well as in AML [163–168]. The roles played by nuclear Akt in cancer have not been investigated in detail so far, however, it would seem logical that, once in the nucleus, Akt could be involved in the regulation of signaling pathways involved in neoplastic cell proliferation and survival. This theory is supported by the finding that the promyelocytic leukemia protein (PML) tumor suppressor is involved in dephosphorylating p-Akt inside the nucleus [169]. PML resides in a special class of nuclear bodies, referred to as PML bodies. PML bodies are involved in numerous functions that include all kinds of protein modifications (acetylation/deacetylation, ubiquitination/deubiquitination, phosphorylation/dephosphorylation, SUMOylation, etc.) [170]. Even if PML is better known for the role it plays in the pathogenesis of acute promyelocytic leukemia (a subset of AML, see [171]), it has been demonstrated that PML expression is reduced in prostate and colon cancer [172]. $PML^{-/-}$ murine embryo fibroblasts (MEFs) displayed a more evident nuclear Ser 473 p-Akt accumulation after serum stimulation than $PML^{+/+}$ MEFs. Also, the invasive prostate cancer cells of $PML^{-/-}$ mice showed a strong nuclear localization of p-Akt. Accordingly, 80% of $PML^{-/-}$ MEFs had cytoplasmic FOXO3a whereas 70% of wild-type cells still retained FOXO3a in the nucleus [169]. Importantly, an Akt-insensitive FOXO3a mutant retained its nuclear localization in $PML^{-/-}$ MEFs. The reduction in nuclear FOXO3a localization correlated with the inactivation of the transcription of three genes that are under the control of the Akt-FOXO3a signaling, i.e. pro-apoptotic Bim, the cell cycle inhibitor $p27^{Kip1}$, and the DNA repair protein, Gadd45 [169]. Considering that: A) okadaic acid (an inhibitor of PP2A) did not affect the levels of nuclear Thr 308 p-Akt in $PML^{-}/^{-}$ MEFs, whereas it did in $PML^{+}/^{+}$ MEFs; B) the A and C subunits of PP2A interacted with PML and localized in PML bodies of $PML^{+}/^{+}$ MEF; and C) PP2A A and C subunits were excluded from the nucleus of $PML^{-}/^{-}$ MEFs, it was concluded that PML specifically recruited the Thr 308 p-Akt phosphatase PP2A as well as p-Akt into PML nuclear bodies [169]. Therefore, when PML levels were low, as in some kinds of cancers, nuclear Akt phosphorylation and activity were high. Moreover, it was documented in a mouse model that PML loss markedly accelerated intestinal tumor onset, incidence, and progression in PTEN-heterozygous mutants that displayed only pre-cancerous polyps. Furthermore, $PTEN^{+/-}$ $PML^{-/-}$ and $PTEN^{+/-}$ $PML^{+/-}$ males (but not $PTEN^{+/-}$ mice that only displayed the in situ form of prostate cancer) developed highly invasive cancers, suggesting that prostate epithelial cells were more sensitive to PML status than the intestine [169]. Importantly, staining for Ser 473 p-Akt was clearly increased in the nucleus in polyps, normal colon, and in prostate lesions of $PTEN^{+/-}$ $PML^{-/-}$ mice when compared with $PTEN^{+/-}$ mice. Therefore, even if most emphasis is usually placed on PTEN deletion for explaining carcinogenesis in prostate cancer [173], also PML could play an important role in the control of p-Akt levels and tumor development.

In this context, it seems intriguing that long-term treatment with high concentrations of statins of A549 lung cancer cells, resulted in a decrease in nuclear Thr 308 p-Akt levels [174]. Thus, it has been hypothesized that the cancer prevention effects of statins [175,176], could be somehow related to down-regulation of nuclear p-Akt levels.

It is very interesting that in the prostate, the extent of Akt nuclear localization increased during the progression from normal tissue to low grade prostatic intraepithelial neoplasia (PIN), high grade PIN, and tumor [165]. Furthermore, in prostatic carcinomas the extent of Akt nuclear localization correlated with the Gleason score, which is the most powerful predictor of tumor progression after prostatectomy [177]. This observation suggested that nuclear Akt could be used as an indicator of the prognosis of prostatic cancer. Higher levels of nuclear p-Akt were also observed in invasive head and neck carcinoma cell lines [178] as well as in glioblastomas [179]. Nevertheless, in patients with estrogen receptor- and progesterone receptor-expressing breast carcinomas, patients displaying positivity for nuclear p-Akt had a longer survival than nuclear p-Akt-negative patients [91].

6. Concluding remarks and future perspectives

It has been 15 years since Akt was demonstrated to migrate to the nucleus. Although considerable progress has been made in understanding some of the roles nuclear Akt plays, yet there still are many outstanding key issues that we do need to tackle. For example, we do not know how exactly Akt enters the nucleus. Moreover, some nuclear Akt substrates have been identified ($p21^{Waf1/Cip1}$, $p27^{Kip1}$, p300, acinus, zyxin, etc.) and the functional consequences of their phosphorylation by Akt have been understood. However, Akt phosphorylates other nuclear substrates (lamin A for example, see [180]), but we do not know how Akt-dependent phosphorylation could affect their functions.

Moreover, it is emerging that nuclear Akt could interact with proteins that are not Akt substrates (Ebp1, B23), yet these interactions are of fundamental importance for regulating key processes taking place within the nucleus. Progress has been recently made in the identification of several Akt-binding partners that modulate its activation, regulate its kinase activity, and define its impact on downstream biological responses in other cell districts [181]. Nuclear Akt-interacting molecules, capable of context-dependent regulation of Akt signaling, could have important roles in controlling signal transduction pathways under both physiological and pathological conditions. Therefore, the identification of additional nuclear Akt-interacting partners may provide a unique opportunity to better understand the mechanisms that modulate Akt signaling activity in the nucleus.

As stated above, Akt is involved in several different disorders. Therefore, it is presently regarded as a potential target for the development of innovative therapies. In some cases, such as cardiomyopathies, it would see desirable to increase intra-nuclear Akt activity. Thus, the possibility of selectively overexpressing Akt within the nucleus appears particularly promising for the therapy of heart disorders, as Akt is a powerful cardioprotective kinase with a well-established capacity to prevent a variety of cardiomyopathic injuries [182].

In case of anti-cancer therapies, the opposite is true, i.e. it could very important to develop strategies to selectively block increased nuclear Akt activity. Indeed, the selective inhibition of nuclear Akt could avoid one of the most feared side effects of Akt activity down-regulation through pharmacological inhibitors that is the blocking of insulin-regulated glucose transport which results in hyperglycemia and type 2 diabetes [183]. In this context, the data demonstrating the possibility of synthesizing Akt inhibitors that could be selectively targeted to the nucleus appear particularly intriguing [158]. Also the possibility of targeting key interactions between Akt and its nuclear partners, could provide yet another opportunity for modulating Akt activity in the nucleus while sparing its functions in other cell districts.

These lines of evidence lead us to believe that dissecting the complexity of nuclear Akt signaling networks will be highly rewarding, as

it could undoubtedly offer valuable insight into the development of novel therapeutic treatments for several disorders, including cancer.

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