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REVIEW

The role of Plk3 in oncogenesis

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The polo-like kinases (Plks) encompass a family of five serine/threonine protein kinases that play essential roles in many cellular processes involved in the control of the cell cycle, including entry into mitosis, DNA replication and the response to different types of stress. Plk1, which has been validated as a cancer target, came into the focus of many pharmaceutical companies for the development of small-molecule inhibitors as anticancer agents. Recently, FDA (Food and Drug Administration) has granted a breakthrough therapy designation to the Plk inhibitor BI 6727 (volasertib), which provided a survival benefit for patients suffering from acute myeloid leukemia. However, the various ATP-competitive inhibitors of Plk1 that are currently in clinical development also inhibit the activities of Plk2 and Plk3, which are considered as tumor suppressors. Plk3 contributes to the control and progression of the cell cycle while acting as a mediator of apoptosis and various types of cellular stress. The aberrant expression of Plk3 was found in different types of tumors. Recent progress has improved our understanding of Plk3 in regulating stress signaling and tumorigenesis. When using ATP-competitive Plk1 inhibitors, the biological roles of Plk1-related family members like Plk3 in cancer cells need to be considered carefully to improve treatment strategies against cancer.

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INTRODUCTION

The strategy of targeting mitosis to fight the progression of rapidly dividing cancer cells is a mainstay of the war against cancer.[1,2] Microtubule-targeting agents that are prime examples for this concept are rather non-selective in their action.[3,4] The aim of modern chemotherapy is to design a more specific, personalized tailored cancer treatment by 'matching the right drug to the right patient at the right time'. Now novel targeted approaches aiming at the inhibition of protein kinases that regulate mitosis are showing significant progress.

Polo-like kinases (Plks) represent a family of highly conserved serine–threonine kinases that play essential roles in cell cycle progression and in the cellular response to different types of stress. Since the discovery of *polo*, a key regulator of mitotic and meiotic events in *Drosophila melanogaster*,[5,6] intensive research efforts have been devoted to the functional analysis of Plks in an enormous phylogenetic space.[7–12] For the five mammalian Plk family members that have been identified to date, the nomenclature of Plk1, Plk2, Plk3, Plk4 and Plk5 for Plk1, serum-inducible kinase (Snk), FGF-inducible kinase (Fnk)/proliferation-related kinase (Prk), Snk/Plk-akin kinase (Sak) and Plk5, respectively, was adopted. Plk1 represents an excellent prognostic marker and target for cancer therapy because of its strong expression in cancer tissues but only weak expression in rare normal tissues. Because of its global involvement in multiple stages of the cell cycle and due to the addiction of cancer cells, numerous functional aspects of Plk1 have been investigated thoroughly in biological, medical and pharmacological research.[13–20] BI 6727 (volasertib) is the lead agent in category of Plk inhibitors, because it is the most advanced compound in clinical development. In a recent report describing the results of a phase II trial, a combination of volasertib and low-dose cytarabine demonstrated an objective response of 31% (13 of 42 patients) compared with just 13.3% (6 of 45 patients) in the low-dose cytarabine monotherapy arm; a response was defined as complete remission or complete remission with incomplete blood count recovery.[21,22] The median overall survival for volasertib plus low-dose cytarabine was 8.0 months as compared with 5.2 months for low-dose cytarabine alone (hazard ratio = 0.63, 95% confidence interval 0.40–1.00; $P = 0.047$). Because of these promising results, a phase III trial was started, investigating this combination in acute myeloid leukemia patients aged 65 and older who are often ineligible for the standard treatment option for acute myeloid leukemia, intensive remission induction therapy. As a consequence of this, the FDA (Food and Drug Administration) awarded breakthrough therapy and orphan drug designations to volasertib to further endow the clinical development of this Plk inhibitor for patients with acute myeloid leukemia. Hence, the expectations are high for late-stage clinical testing that might lead to the first approved agent in this class.

Despite the promising clinical development of volasertib, it should be considered that all Plk1 inhibitors that are currently in clinical trials are ATP-competitive compounds that target multiple members of the superfamily of protein kinases. Since protein kinases are highly related in sequences and three-dimensional structures, especially in their ATP-binding region, it is extremely challenging to develop Plk1-specific inhibitors. Although being highly selective, volasertib potently inhibited Plk1 as well as the two closely related kinases Plk2 and Plk3 (half maximal inhibitory concentration (IC_{50}) values 0.87, 5 and 56 nmol/l, respectively;[23] Table 1). Additional Plk inhibitors currently being tested in clinical trials, including BI 2536, NMS-P937 and GSK461364A, also target additional Plk family members including Plk3[24–26] (Table 1).

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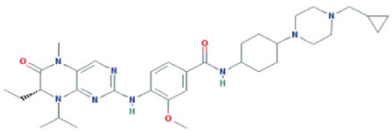
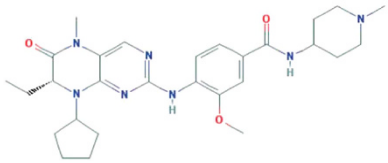
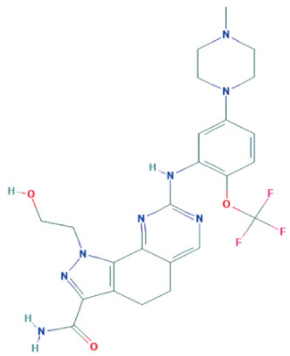
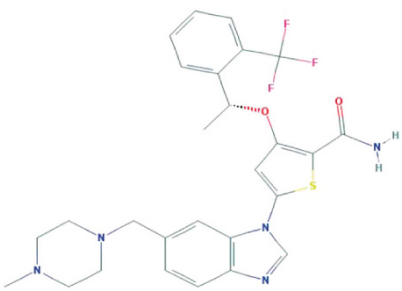
Table 1. Plk1 inhibitors

Inhibitor	*Structure*	*IC_{50} (Plk1), nM*	*IC_{50} (Plk2)*	*IC_{50} (Plk3)*	*References*
BI 6727, volasertib		0.87	5 nM	56 nM	23
BI 2536		0.83	3.5 nM	9.0 nM	24
NMS-P937, UNII-67RM91WDHQ		2	10 μM	10 μM	25
GSK461364A		0.5	860 nM	1000 nM	26

Abbreviation: Plk, polo-like kinase. The two-dimensional structures of chemical compounds were taken from database PubChem, http://pubchem.ncbi.nlm.nih.gov.

Although Plk1 received the most attention in biological, medical and pharmaceutical research,[27–31] the functions of the other members of the Plk family are less explored. Here, we survey the progress in understanding the role of Plk3 in mammalian cells and in cancer. The fine understanding of Plk3 could help to improve treatment strategies against cancer when using ATP-competitive inhibitors of Plk1 that have the potential to target multiple members of the Plk family.

EXPRESSION OF PLK3 IN CELLS AND TISSUES

Originally mouse Plk3 was described as an immediate early gene, which shows peak expression at 1 h following the stimulation of quiescent cells with growth-promoting agents due to transcriptional activation.[32] However, murine Plk3 mRNA levels are only transiently elevated for a period of ~8 h. Although the Plk3 mRNA can be found in numerous fetal and newborn mouse tissues, it is restricted only to hematopoietic tissues, thymus, placenta, ovaries and testes in adult tissues. Stimuli that produce synaptic plasticity in the rat brain, including those that evoke long-term potentiation, dramatically increase the levels of Plk2 and Plk3 mRNAs.[33] Phosphorylation of α-synuclein on Ser-129 is one of the major post-translational modifications found in Lewy bodies, the typical pathological hallmark of Parkinson's disease.[34] It was found that both Plk2 and Plk3 phosphorylate α-synuclein on Ser-129.[35–37]

In human A549 cells, that were arrested at different stages of the cell cycle, low levels of the Plk3 transcript were found in the M and G_1 phases with peak levels detected during the late S and G_2 stages[38] (Figure 1a). It should also be noted that Plk3 transcripts of different sizes were detected in mouse and human cells.[32,39]

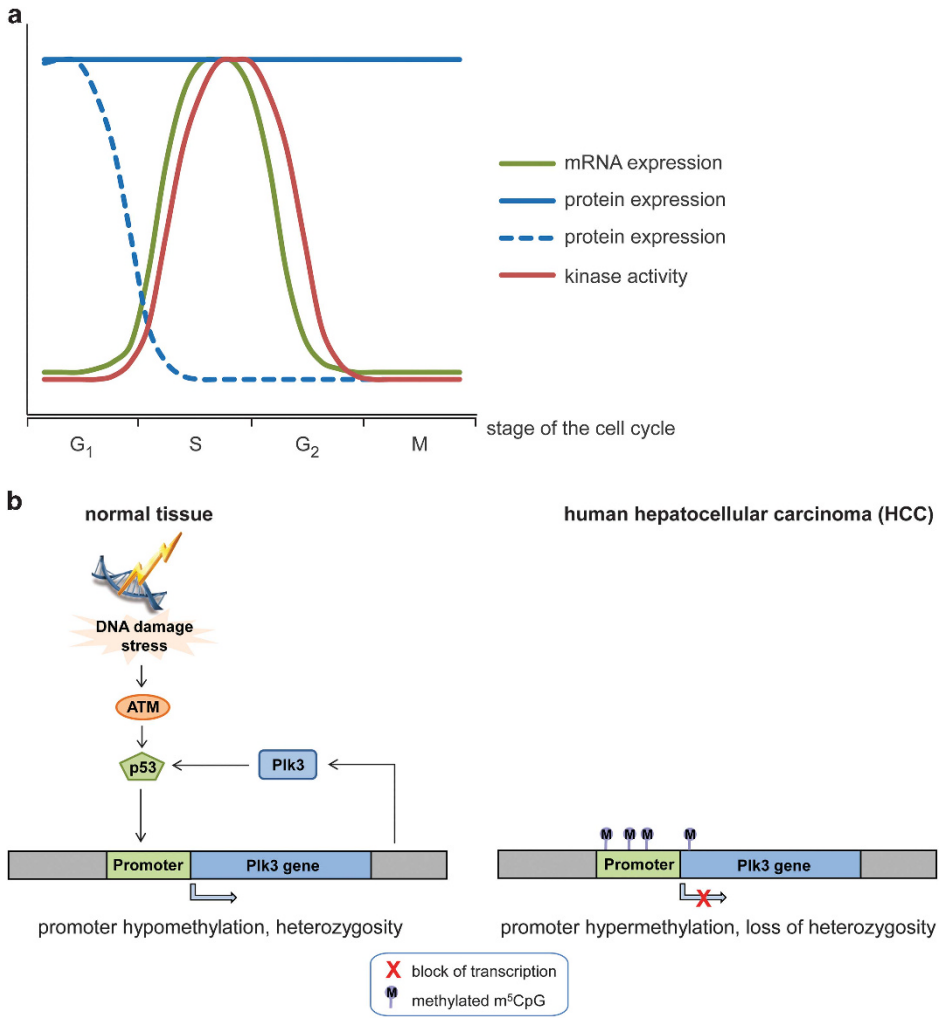


Figure 1. The expression of Plk3 during the cell cycle and its transcriptional regulation in synchronized exponentially growing cells. (**a**) Plk3 expression (mRNA, protein) and kinase activity throughout the cell cycle. Plk3 mRNA expression level, which is low during G_1 and M, peaks at late S and S/G_2 stages[38] (green line). Many reports provide evidence for an expression of Plk3 protein throughout the cell cycle[38,41,42,142] (blue line). It was also demonstrated that Plk3 protein expression is restricted to the G_1 phase[43] (dashed blue line). Although the Plk3 kinase activity is low during mitosis, G_1 and G_1/S phases, it increases during late S and G_2 stages[38] (red line). (**b**) Transcriptional regulation of Plk3. In response to ionizing radiation (IR), p53 binds to the Plk3 promoter and induces its expression. Plk3 phosphorylates and activates p53. Human hepatocellular carcinoma (HCC) shows in comparison to nonneoplastic liver tissue very low levels of Plk3 mRNA and protein expression because of Plk3 promoter hypermethylation and/or loss of heterozygosity at the Plk3 gene loci.

Human Plk3 transcripts exhibiting a length of 2.4 kb were most abundant in the placenta and lung.[40] A lower frequency of human Plk3 transcripts was observed in skeletal muscle, heart, pancreas and kidney, while weak expression was detected in the liver and brain. In contrast to Plk1 expression that was detected only in tissues with a high mitotic index,[27] Plk3 expression does not correlate with the proliferative activity of cells.[40]

Remarkably, several controversial publications exist that describe the expression pattern during the cell cycle and the subcellular localization of Plk3. Several reports suggest that the abundance of the Plk3 protein varies, but it is present throughout the cell cycle[38,41,42] (Figure 1a). In our own experiments using different Plk3-specific antibodies we could confirm this. In contrast, in cycling HeLa cells synchronized by double thymidine block, the presence of the Plk3 protein was reported to be restricted to the G_1 phase concurrent with the expression of cyclin D1[43] (Figure 1a). The kinase activity of Plk3 is low during G_1/S reaching the peak level during late S and G_2 phases with moderate levels at metaphase in synchronized A549 cells[38] (Figure 1a). Remarkably, different groups found the protein to reside at different locations: at the nucleolus,[43] centrosomes, spindle microtubules,[44] Golgi apparatus[45] and cellular membrane.[40] Taken together, reports of the localization of Plk3 may vary depending on the cell lines, and whether the investigation was carried out on endogenous or ectopically expressed full-length or truncated Plk3. Since one report suggested that this conflict is primarily due to the poor solubility of the endogenous Plk3 protein and the suboptimal antibody specificity,[43,46] we re-evaluated most of the commercially available antibodies (BD (Becton, Dickinson and Company), Franklin Lakes, NJ, USA; Abcam, Cambridge, UK) on full-length and truncated forms of Plk3 to specify the binding domain of the antibodies within Plk3. We found that the antibodies tested are directed against the N-terminal portion of Plk3 (Abcam) or against the interdomain that connects the kinase domain with the polo-box domain (PBD; BD). Both regions show a low degree of conservation within the family of Plk and are therefore very well suited for the generation of antibodies. In addition, we found that the Plk3 protein, which is present predominantly in the cytoplasm, at the membrane and to a lesser extent in the nucleus, differ in size. To elucidate whether the different forms of Plk3 represent isoforms of varying lengths or different post-translationally modified proteins as indicated by

Table 2. Substrates of Plk3

Protein	*Residue*	*Method*	*References*
14-3-3 Epsilon	T208	*In vitro*	89
ATF-2	T69	*In vitro*	123
ATF-2	T71	*In vitro*	123
Bcl-xL	S49	*In vivo, in vitro*	91
CALU	S44	*In vitro*	89
CALU	T60	*In vitro*	89
CALU	T177	*In vitro*	89
CALU	T196	*In vitro*	89
CALU	T254	*In vitro*	89
CCNB1	S133	*In vitro*	90
CDC25A	T80	*In vivo, in vitro*	92
CDC25A	S513	*In vitro*	103
CDC25A	S519	*In vitro*	103
CDC25C	S191	*In vitro*	93
CDC25C	S198	*In vitro*	90,93
Chk2	S62	*In vitro*	98
Chk2	S73	*In vitro*	98
HIF1A	S576	*In vivo, in vitro*	119
HIF1A	S657	*In vivo, in vitro*	119
HSP90B	S718	*In vitro*	89
Jun	S63	*In vivo*	85
Jun	S73	*In vivo*	85
NPM1	S4	*In vitro*	94
p53	S20	*In vivo, in vitro*	59,56,48
p73α	aa 63–113	*In vitro*	115
PTEN	T366	*In vivo, in vitro*	116
PTEN	S370	*In vivo, in vitro*	116
SNCA	S129	*In vivo, in vitro*	35,36
SNCB	S118	*In vivo, in vitro*	36
TOP2A	T1343	*In vivo, in vitro*	90
VRK1	S342	*In vivo, in vitro*	95

Abbreviation: Plk, polo-like kinase. Data were taken from database PhosphoSitePlus, www.phosphosite.org.[96]

different reports, further more detailed studies are required. In addition, it remains to be investigated, whether the type of Plk3 antibody used for previous immunofluorescence studies of cancer tissues and cell lines determined the staining pattern, that is, the identification of Plk3 at different subcellular structures.

The ectopic expression of either the full-length Plk3 or only its PBD induces chromatin condensation and apoptosis.[47–49] However, Plk3-depleted cells synchronized by serum starvation or exponentially growing cells that were efficiently depleted of Plk3 were found to be arrested in G_1/G_0 and failed to enter the S phase indicating its requirement for cell cycle progression.[43] Cells with reduced Plk3 also had a significantly reduced level of cyclin E protein, although the levels of cyclin D and cell division cycle 25A (CDC25A) appeared to be unaffected. This shows that Plk3 attenuates cyclin E expression, and by doing so, may regulate the G_1 restriction point. It also demonstrates that Plk3 is required for the continued cell cycling under permissive conditions. Remarkably, Plk3 can complement the mitotic defects associated with a temperature-sensitive mutation in *Saccharomyces cerevisiae* cell division cycle 5 (CDC5) suggesting that Plk3 may be a bona fide mammalian polo/CDC5 homolog with overlapping functional properties.[50]

DOMAIN ORGANIZATION, FUNCTION AND REGULATION OF PLK3

Regulation at the transcriptional level

Plk3 has been described as an ionizing radiation (IR)-responsive gene[51,52] that is transcriptionally regulated by p53.[53–55] In response to IR, p53 binds to the Plk3 promoter and induces its expression[55] (Figure 1b). In cells with a functional p53, 4 h after exposure to IR, the p53 occupancy of the Plk3 promoter peaks corresponding with the mRNA level of Plk3. Twenty-four hours post irradiation, the transcript levels of Plk3 remained elevated whereas the level of promoter bound p53 declines. Following radiation exposure, p53 binding and subsequent transcriptional activation of Plk3 gene were significantly diminished in cells expressing either mutant p53 or, in ataxia telangiectasia mutated (ATM)-deficient cells, displaying impaired p53 activation.[55] As p53 phosphorylation by Plk3 following DNA damage and oxidative stress promotes p53 activity,[48,56,57] induction of Plk3 expression by p53 provides evidence for a reciprocal regulatory mechanism to amplify signals in p53-related stress response.[55]

In human hepatocellular carcinoma (HCC), Plk3 downregulation is linked to promoter hypermethylation and/or loss of heterozygosity at the Plk3 gene loci[58] (Figure 1b). Plk3 mRNA expression gradually declines from nonneoplastic liver tissue to HCC with the lowest levels being detected in HCC with poor survival.[58]

Doxycycline/superoxide stimulation of cells induces Plk3 expression and leads to the phosphorylation of p53 on Ser-20 in a Plk3-dependent manner[59] (Table 2). An nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) binding site was identified in the promoter of Plk3, which is required for the induction of Plk3 by the v-rel avian reticuloendotheliosis viral oncogene homolog A (RelA)-NF-κB complex, indicating that Plk3 is a RelA-NF-κB-regulated gene.[59] RelA is the subunit of NF-κB transcription factors, and is a key regulator of antiapoptotic and proapoptotic responses. Overexpression of wild-type Plk3 in HCT116 $p53^{+/+}$ colon cancer cells induced rapid apoptosis, whereas the overexpression of wild-type Plk3 in HCT116 $p53^{-/-}$ cells or of the kinase-defective mutant $Plk3^{K91R}$ in $p53^{+/+}$ cells led to a delayed onset of apoptosis, for which the amino-terminal domain (amino acids 1–26) of Plk3 is required.[59] Furthermore, the depletion of Plk3 by RNAi suppressed doxycycline/superoxide-mediated apoptosis.[59] These data suggest that Plk3 is a RelA-NF-κB-regulated kinase that mediates both p53-dependent and -independent proapoptotic signaling pathways.

Studies of tristetraprolin (TTP)-deficient mice and cells have identified the Plk3 transcript as a target of TTP[60] demonstrating that Plk3 function is also regulated at the level of mRNA stability.[61] TTP is a zinc-finger protein harboring a tandem motif with three conserved cysteine residues and one histidine residue (CCCH). It is rapidly induced by mitogens in mouse embryonic fibroblasts (MEFs).[60] TTP can bind to class II AU-rich elements in the 3'-untranslated region (UTR) of the mRNA leading to the removal of the polyadenylated tail from the mRNA and consequently its degradation.[62] After serum stimulation, the stability of Plk3 mRNA was enhanced through the slowdown of Plk3 transcript decay in TTP-deficient murine MEFs in comparison to wild-type fibroblasts.[61] The short half-life of the Plk3 mRNA in MEFs with intact TTP is consistent with the presence of conserved AU-rich elements within the Plk3 3'-UTR.[32,60] The three AU-rich elements present in Plk3 transcript are essential for both the binding of TTP to the 3'-UTR and promoting the mRNA degradation in transiently transfected HEK293 cells.[61] The modulation of the stability of Plk3 mRNA by TTP may contribute to the regulation of cellular processes, in which Plk3 is involved, such as cell cycle progression, apoptosis, stress response and carcinogenesis.

Regulation at the protein level

Members of the Plk family are characterized by a classical serine/threonine kinase domain located within the N-terminal half of the molecule and the C-terminal PBD, which is unique to the Plk family (Figure 2a). The PBDs of Plk1–3, which are made of two polo-box subdomains (PB1 and PB2), share a similar architecture. The PBD of human Plk1 regulates its catalytic activity, localization and substrate binding. This domain constitutes a binding pocket for the accommodation of phosphorylated Ser or Thr residues of

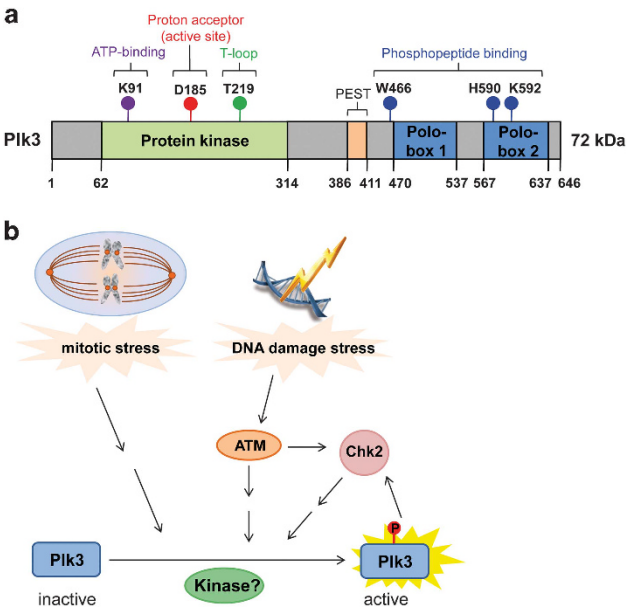


Figure 2. Domain organization and post-translational regulation of Plk3. (**a**) Domain architecture and key residues. Plk3 contains an amino-terminal kinase domain and a carboxy-terminal PBD (polo-box domain) consisting of two polo-boxes. Asp-185 is the proton acceptor site, which could serve as the key catalytic residue of the kinase domain. Lys-91 belongs to the ATP-binding site. Thr-219 is located within the activation loop or T-loop of the kinase domain corresponding to Thr-210 in Plk1. Phosphorylation of conserved residues within T-loop is a common mechanism of activation for Plk1[75,143] and Plk2.[144] Trp-466, His-590 and Lys-592 are potential phosphopeptide-binding sites in Plk3 corresponding to Trp-414, His-538 and Lys-540 in Plk1, respectively. Plk3 contains a predicted proline–glutamate–serine–threonine (PEST) motif in the linker region (Epestfind, http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind).[43] (**b**) Post-translational regulation of Plk3. Following DNA damage or mitotic stress, Plk3 becomes phosphorylated by an unknown kinase leading to Plk3 activation. Whereas the DNA damage-induced activation of Plk3 is ATM-dependent and mediated by Chk2, the mitotic stress-induced activation is ATM-independent.

other proteins.[63–65] Thus, a 'priming phosphorylation' by 'Pro'-directed kinase like cyclin-dependent kinase 1 (CDK1) or mitogen-activated protein kinases often precedes the binding of Plk1 to its targets. The optimal phosphopeptide-binding motif for the PBDs from different members of the human Plk family was determined by oriented peptide screening.[65] The PBDs of Plk1–3 selected distinct but overlapping motifs and all showed uniequivocal selection for Ser in the pThr-1 position. Remarkably, although the PBDs of Plk1-5 (homologies of the PBDs compared to the PBD of Plk3: Plk1, 37.3%; Plk2, 48.5%, Plk4, 21.8% and Plk5, 37.3%) show a considerable divergence in amino acid sequence and the corresponding human Plks fulfill different functions, their PBDs recognize similar motifs.[65] To answer the question whether this similarity indicates that Plk1–3 share an overlapping set of target proteins, future studies including proteomic analyses are required that will compare the selectivity profiles of the PBDs belonging to Plk1–3.

Plk1 is spatially regulated through the targeting activity of the conserved PBD. The current prevailing model is that the PBD binds to a phospho-epitope generated by CDK1 or other Pro-directed kinases. In addition, Plk1 self-promotes its localization by generating its own PBD-docking site.[66] In addition to the non-self-priming and self-priming mechanisms that regulate the localization of Plk1, recent evidence suggests that the *D. melanogaster* counterpart *polo* binds via its PBD to a non-phosphorylated target, the microtubule-associated protein 205 (Map205^{PBM}).[67] The role of a priming phosphorylation for the localization of Plk3 is hitherto unknown.

Exogenous murine Plk3, which shuttles between the cytoplasm and the nucleus, is rapidly degraded via the ubiquitin-proteasome pathway in the nucleus.[68] The nuclear export of Plk3 is chromosome region maintenance 1 protein homolog (CRM1) dependent. Overexpressed kinase-deficient Plk3 (Plk3-K92M) does not enter the nucleus as efficiently as wild-type Plk3 does and consequently it is significantly more stable indicating that the catalytic activity of Plk3 is required for its efficient nuclear import.[68] Whether the enzymatic activity of human endogenous Plk3 plays a similar regulatory role remains to be elucidated. Human Plk3 contains predicted PEST (Pro-Glu-Ser-Thr) sequences that could promote its degradation through the Skp1–Cul1–F-box-protein (SCF) ubiquitin ligase[43] (Figure 2a). However, constitutively active and kinase-inactive human Plk3 were expressed at similar levels in A549 cells.[44]

Post-translational regulation

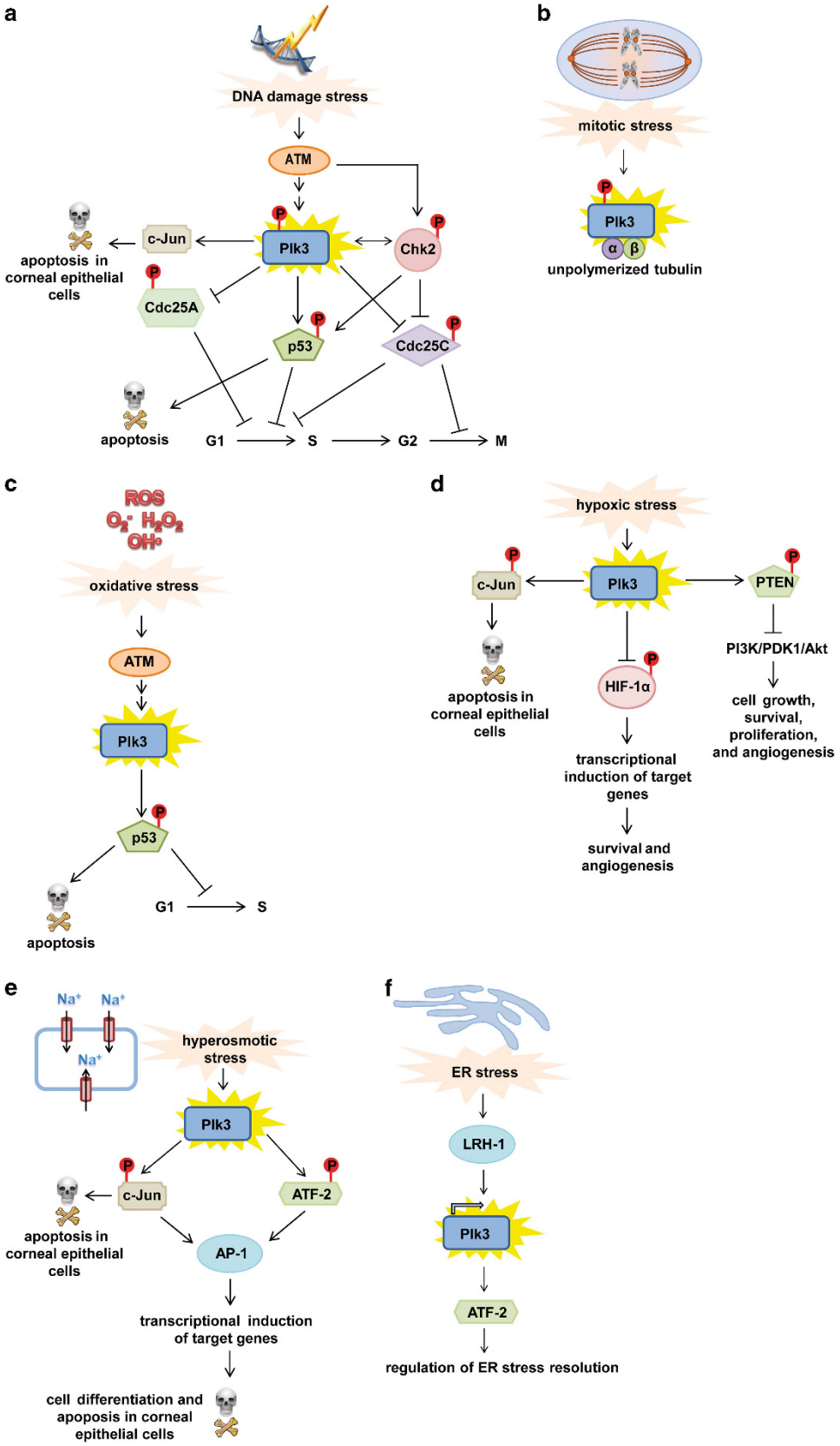
Binding of the PBD to the kinase domain inhibits the enzymatic activity of Plk1 and also reduces the affinity of the PBD to phosphorylated peptides.[64,65,69] The recently published crystal structure of the complex of the kinase domain of the zebra fish Plk homolog and the PBD together with a PBD-binding motif of *D. melanogaster* Map205^{PBM} that stabilizes the complex, suggests that Plk1 is autoinhibited in the resting state, that is, the PBD binds and reduces the flexibility of the hinge region of the kinase domain in a distinct conformation from that of the phosphopeptide-bound PBD.[67,70] This excellent study sheds new light on the mechanism behind the activation of Plk1. The kinase domain can be partially or fully activated through phosphorylation of Thr-210 and Ser-137 and binding of a specific phosphopeptide independently or sequentially at different stages of the cell cycle or at different subcellular locations. In human cells, Aurora A with the help of its cofactor Bora phosphorylates Plk1 within its T-loop at Thr-210, which supports entry into mitosis during the G_2/M transition in a normal cell cycle and after DNA damage checkpoint arrest.[71–74] The kinases that regulate the activation of Plk3 have not been identified yet. Although a phospho-mimicking mutation at Thr-210 leads to the activation of Plk1,[75] the replacement of Thr-219, which represents the corresponding site in Plk3, by a negatively charged residue, surprisingly reduced the Plk3-specific activity[49,76] (Figure 2a). Hence, structural studies of Plk3 are needed to clarify its regulation and mechanism of action.

Mitogenic stimulation of serum-starved quiescent cells with fetal calf serum resulted in a transient modification of murine Plk3 suggesting a functional change during the entry of cells into the cell cycle from quiescence.[41] In cycling cells, the murine Plk3 protein becomes phosphorylated as cells cross the G_2/M border and gets dephosphorylated in late M phase indicating a critical function of Plk3 during mitosis.[41] However, a phosphorylation of human Plk3 during the cell cycle progression has not been detected yet.[42]

Following DNA damage, Plk3 was phosphorylated and activated in an ATM-dependent manner, but Plk3 was not directly phosphorylated by ATM *in vitro* [42,48,56] (Figure 2b). The interaction between Plk3 and checkpoint kinase 2 (Chk2), a multifunctional enzyme whose functions are central to the induction of cell cycle arrest and apoptosis by DNA damage, was shown to be p53-independent and was enhanced upon DNA damage.[57] Chk2 stimulated Plk3 kinase activity *in vitro* and the ectopic expression of Chk2 resulted in the activation of cellular Plk3 indicating that Chk2 may mediate the direct activation of Plk3 in response to genotoxic stresses[57] (Figure 2b). It remains unclear, whether Plk3 is a direct target of Chk2 *in vivo*.

In contrast to DNA damage stress, Plk3 was partially phosphorylated in response to nocodazole-mediated spindle

disruption independent of ATM activity (Figure 2b). A fast protein liquid chromatography-based study revealed that while the phosphorylated portion of Plk3 from nocodazole-treated cells formed complexes of ~150 and 600 kDa, and greater with unknown cellular components, the unphosphorylated portion was eluted in fractions between 400 and 500 kDa.[42]



The hypophosphorylated form of Plk3 from exponentially growing cells was eluted with a molecular weight between 140 and 220 kDa.

Mass spectrometry-based analyses of the proteome-wide phosphorylation pattern revealed the phosphorylation of Plk3 at Tyr-136 in human erythrocyte membrane;[77] Thr-348 in HEK293 cells;[78] Ser-371 and Ser-381 in mouse embryonic stem cells;[79] Thr-503, Tyr-506 and Ser-510 in mitotic HeLa cells;[80] Thr-614 and Tyr-615 in human osteosarcoma (MNNG-HOS) cells.[81] The kinases that are responsible for the Plk3 phosphorylation and the functional consequences are yet unknown.

THE ROLE OF PLK3 IN GENOTOXIC AND NON-GENOTOXIC STRESS PHENOTYPES

In the original model of carcinogenesis Hanahan and Weinberg[82] had proposed six hallmarks of cancer that collectively promote survival, proliferation and apoptotic resistance in foreign environments. An additional attribute 'evading immune surveillance' was added to the model of cancer development by Kroemer and Pouyssegur.[83] The current model of carcinogenesis was extended with the addition of five further, equally prevalent carcinogenic stress characteristics based on recent analyses of cellular phenotypes by Elledge and colleagues.[84] These stress phenotypes (DNA damage/replication, proteotoxic, mitotic, metabolic and oxidative stress) of cancers are not responsible for initiating tumorigenesis, but they are common features of many tumor types. Plk3 was shown to be a stress response protein that is involved in the stress-induced signaling pathways in cancer cell lines of various origins and in primary normal cells.[42,48,57] Multiple studies that investigated the role of Plk3 under various stress conditions could demonstrate that the level of the Plk3 protein remain stable throughout the cell cycle,[42] whereas its transcript levels and protein kinase activity are upregulated.[39,42,48,55–57,85–87]

DNA damage stress

P53 is a key regulator of cellular stress and an important tumor suppressor. p53 is activated upon different types of cellular stress and integrates the incoming signals so that an appropriate cellular response is made. An increasing number of p53-target genes with a growing functional complexity in the cell, are being identified, and many transcription-independent functions of p53 have also been described.[88] In a study on IR-dependent p53 binding and subsequent transcriptional activation of genes using both *in vitro* and *in vivo* assays various novel p53 targets including Plk3 were found.[55] An analysis of gene expression signatures in HCT116 and HCT116TP53$^{-/-}$ colon cancer cells identified multiple p53-target genes including Plk3 that were associated with four microenvironmental components of the inflammatory response ($NO^{\bullet}$ (nitric oxide), H_2O_2 (hydrogen peroxide), DNA replication arrest and hypoxia).[53] Several putative p53-responsive elements were found within the promoter region of Plk3 based on a novel position weight matrix method.[53] Moreover, in response to DNA damage, the interaction between Plk3 and the tumor-suppressor protein p53 is enhanced, whereby Plk3 phosphorylates Ser-20 of p53 in an ATM-dependent manner (Table 2). To what extent Plk3 phosphorylates additional targets like 14-3-3 epsilon, B-cell lymphoma-extra large (Bcl-xL), G_2/mitotic-specific cyclin B1 (CCNB1), cell division cycle 25A (CDC25A), cell division cycle 25C (CDC25C), nucleophosmin/nucleoplasmin family member 1 (NPM1) and vaccinia related kinase 1 (VRK1) following DNA damage requires future analysis[89–96] (Table 2). This event contributes to the activation of the DNA damage checkpoint and cell cycle arrest and/or apoptosis suggesting that Plk3 links DNA damage to cell cycle arrest and apoptosis through the ATM/p53 pathway[48] (Figure 3a).

The investigation of IR-induced changes in the global gene expression of primary human fibroblasts using a microarray analysis algorithm EPIG (extracting microarray gene expression patterns and identifying biologically significant genes) demonstrated that many p53-target genes including Plk3 were significantly upregulated at 2 h after the IR.[54] Analysis of gene expression in diploid fibroblasts at 24 h after the IR revealed a profile similar to that of synchronization behind the G_1 checkpoint but with the characteristics of G_0 growth quiescence indicating that changes in gene expression, including the upregulation of Plk3, initiate cell cycle arrest.[54] The Plk3 gene expression profile of mouse white blood cells shows oscillating changes over a broad dose range of 2–8 Gy following IR.[51] Furthermore, in primary human fibroblasts the radiation-induced transcriptional profile showed a rapid increase in Plk3 gene expression after IR with 2 Gy indicating that Plk3 is a radiation response gene.[52] The time- and dose-dependent responses of the Plk3 gene suggests that measurements of the Plk3 transcript level may be a promising biomarker for evaluating radiation exposure doses.

Whereas DNA damage leads to the inhibition of Plk1 associated with G_2 arrest,[97] Plk3 activity increases rapidly. Plk3 is phosphorylated and activated following DNA damage in an ATM-dependent manner, but evidence for a direct phosphorylation of Plk3 by ATM is still missing[42,48,56] (Figure 3a). The binding of Plk3 to Chk2 correlates with increasing p53 activity and this interaction is enhanced upon DNA damage.[57] Although Chk2 stimulates Plk3 kinase activity *in vitro*, the ectopic expression of Chk2 results in the activation of cellular Plk3 indicating that Chk2 might trigger cellular signaling via activation of Plk3 in response to genotoxic stresses[57] (Figure 3a). It could be demonstrated that Plk3 phosphorylates Chk2 primarily at Ser-73 and to a lesser extent at Ser-62 *in vitro*[98] (Table 2). This priming phosphorylation facilitates subsequent phosphorylation of Chk2 on Thr-68 by ATM in response to DNA damage suggesting that Plk3 may also regulate Chk2 activity upon DNA damage. These results indicate that Plk3 is part of a bidirectional feedback mechanism involved in the stress-induced signaling pathways and the regulation of the DNA damage checkpoint.

CDC25A is rapidly degraded in response to DNA damage or stalled replication and is known to be a key substrate in the checkpoint response.[99,100] Ultraviolet and IR treatments activate

Figure 3. Activation of Plk3 in response to different types of stress and its involvement in stress-induced signaling pathways. (**a**) Following DNA damage, Plk3 is activated in an ATM-dependent manner. In addition, Chk2 may stimulate Plk3 kinase activity. Active Plk3 phosphorylates Chk2, p53, CDC25A, CDC25C and c-Jun in HCE (human corneal epithelial cells). The phosphorylation of Chk2, p53 and c-Jun by Plk3 leads to their activation, whereas the phosphorylation of CDC25A and CDC25C results in their inactivation causing cell cycle arrest and/or apoptosis. (**b**) In response to mitotic stress Plk3 is phosphorylated, activated and appears to form complexes with unpolymerized tubulin and other cellular components. (**c**) Oxidative stress induces the activation of Plk3 and the phosphorylation of p53 in an ATM-dependent manner leading to the activation of p53 followed by G_1/S arrest or apoptosis. (**d**) Phosphorylation of HIF-1α and PTEN by Plk3 in response to hypoxic stress leads to the destabilization of HIF-1α and the stabilization of PTEN resulting in reduced cell survival, angiogenesis and proliferation. Hypoxic stress-induced activation of Plk3 and c-Jun phosphorylation by Plk3 leads to growth attenuation and apoptosis in HCE cells. (**e**) Upon hyperosmotic stress, Plk3 is activated in HCE cells and phosphorylates the transcription factors c-Jun and ATF-2 resulting in the activation of the AP-1 transcriptional complex, which leads to cell differentiation and apoptosis. (**f**) Following ER stress, nuclear receptor LRH-1 (liver receptor homolog-1) induces expression of Plk3, which then phosphorylates and activates ATF-2 regulating the ER stress resolution.

the ATM/ATR–Chk1/2 pathway[99,101] leading to the phosphorylation of CDC25A and triggering the signal for its degradation by the proteasome.[99] This has been proposed to result in the inactivation of the CDK2/cyclin E complex that involves a DNA replication arrest. CDC25A phosphorylation and degradation represent a rapid cellular response that imposes a DNA synthesis block prior to the activation of the p53–p21 pathway, which ensures a more sustained proliferation arrest.[102] Plk3 phosphorylates the cell cycle protein phosphatase CDC25A on Ser-513 and Ser-519 *in vitro*[103,104] (Table 2). The depletion of Plk3 in MEFs leads to the reduction of DNA damage-mediated degradation of CDC25A protein indicating that the phosphorylation of CDC25A by Plk3 contributes to the proteasome-mediated degradation of CDC25A following IR (Figure 3a).[103] Plk3 also interacts with and phosphorylates the protein phosphatase CDC25C on Ser-216, a residue that is also phosphorylated by Chk1 and Chk2[105] (Table 2). This phosphorylation leads to the inactivation and the nuclear export of CDC25C into the cytoplasm causing G_2/M arrest in response to DNA damage[106,107] (Figure 3a).

Upon ultraviolet irradiation, activated Plk3 phosphorylates and activates the proto-oncogene c-Jun in corneal epithelial cells resulting in ultraviolet stress-mediated apoptosis[87] (Table 2). c-Jun is a critical component of the activator protein 1 (AP-1) transcription factor that consist of homo- or heterodimers of basic region-leucine zipper proteins that belong to the Jun, FBJ murine osteosarcoma viral oncogene homolog (Fos), activating transcription factor (ATF) and v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (Maf) subfamilies.[108] The variety of dimeric complexes and the dual roles of AP-1 as a transcriptional activator and repressor of genes may explain how c-Jun regulates so many different, and sometimes opposing, cellular processes.[109] For example, consistent with its role in cell proliferation, c-Jun is induced by the transient expression of oncogenes and is required for the transformation of fibroblasts by activated H-Ras.[110–112] Conversely, genetically modified cells have provided evidence that c-Jun is required for mediating the apoptotic response of neurons to stress.[109,113,114] However, the role of c-Jun in regulating apoptosis of corneal epithelial cells remains to be explored in detail.

Plk3 is transcriptionally induced in response to cisplatin, whereas its protein level is kept constant indicating that cisplatin-mediated control of Plk3 stability might contribute to DNA damage-induced apoptosis.[115] Upon DNA damage caused by cisplatin, Plk3 interacts with p73 *in vivo*. Plk3 phosphorylates p73 *in vitro*[115] (Table 2). Plk3 inhibits the transcriptional and proapoptotic activity of p73 as well as decreases its stability in a kinase activity-dependent manner suggesting an important role of Plk3 in the regulation of cisplatin-mediated apoptotic response through the inhibition of p73.[115]

Mitotic stress

Additional observations suggested that Plk3 may be a stress-responsive protein that not only responds to DNA damage but also to mitotic spindle disruption. The amount of Plk3 mRNA, but not that of Plk1 mRNA, is rapidly and transiently increased in response to mitogenic stimulation.[39] Cells incubated with nocodazole arrest in G_2/M. Microscopy of nocodazole-treated cells shows that they do enter mitosis but cannot form metaphase spindles because microtubule dynamics is perturbed. The absence of microtubule attachment to kinetochores activates the spindle assembly checkpoint causing the cell to arrest in prometaphase. In response to nocodazole administration, Plk3 becomes phosphorylated independent of ATM activity and appears to become associated and form complexes with other cellular components.[42] The phosphorylated Plk3 co-immunopreciptated with unpolymerized tubulin in fractions containing complexes suggesting a role in the nocodazole-mediated arrest (Figure 3b). The activation and phosphorylation of Plk3 in response to different cellular insults, for example, DNA damage and mitotic spindle disruption suggest that Plk3 might serve as a stress response gene and participate in G_2 and G_2/M checkpoints. Phosphorylation of tumor-suppressor proteins like Chk2,[98] p53[48,56] and phosphatase and tensin homolog (PTEN)[116] by Plk3 may also contribute to checkpoint control signaling (Table 2).

Oxidative stress

In both tumor development and responses to anticancer therapies the control and regulation of oxidative stress are critical elements. Many signaling pathways that contribute to carcinogenesis can also regulate the metabolism of reactive oxygen species through different mechanisms. High reactive oxygen species levels are generally disadvantageous for cells, and the redox status of cancer cells usually differs from that of normal cells. Because of metabolic and signaling aberrations, cancer cells exhibit elevated reactive oxygen species levels. Analysis of global changes in gene expression induced by oxidative stress *in vivo* in the liver of $Sod1^{-/-}$ (cytosolic superoxide dismutase knock-out (KO)) mice revealed an upregulation of Plk3 and other p53-target genes pointing to the role of p53 in the induction of Plk3 gene expression in response to oxidative stress.[117] In addition, Plk3 is rapidly activated by reactive oxygen species in normal diploid fibroblast cells (WI-38) correlating with a subsequent increase in p53 protein level, which indicates that p53 may be a direct target of Plk3 during stress response.[57] Oxidative stress, for example, exposure to H_2O_2 (hydrogen peroxide) induces the activation of Plk3 and the phosphorylation of p53 on Ser-20 in an ATM-dependent manner leading to the activation of p53 followed by the induction of p21 (Figure 3c; Table 2).[56] Different studies strongly suggest that Plk3 is involved in the oxidative stress-induced phosphorylation of p53 on Ser-20 and its subsequent activation.[56]

Hypoxic stress

Low tissue oxygen levels, known as hypoxia, are a common feature of solid tumors and may provide an opportunity for the development of effective anticancer drugs. Recent analyses have revealed complex interconnections between oncogenic activation, hypoxia signaling systems and metabolic pathways that are dysregulated in cancer.[118] $Plk3^{-/-}$ MEFs show increased expression of hypoxia-inducible factor 1-alpha (HIF-1α) compared to $Plk3^{+/+}$ MEFs and a hypersensitivity to the induction of HIF-1α upon hypoxia.[76] Hypoxia-induced HIF-1α expression was tightly associated with a significant downregulation of Plk3 expression in HeLa cells.[76] Plk3 interacts with HIF-1α under hypoxia and phosphorylates it on Ser-576 and Ser-657 *in vitro*[119] (Table 2). Furthermore, the ectopic expression of Plk3 suppresses the nuclear accumulation of HIF-1α induced by nickel or cobalt ions.[76] Moreover, Plk3-mediated phosphorylation leads to the destabilization of HIF-1α *in vivo*[119] (Figure 3d). The fact that HIF-1α is a key player in activating cell survival and angiogenesis during malignancy, and Plk3 is a negative regulator of HIF-1α stability, indicate that enhanced tumorigenesis in Plk3-null mice could at least be partly mediated by a deregulated HIF-1α pathway.

Although Plk1 was shown to phosphorylate PTEN and neural precursor cell expressed developmentally down-regulated 4-1 (Nedd4-1), an E3 ubiquitin ligase of PTEN, which results in PTEN inactivation,[120] the activity of Plk3 seems to have the opposite effect: following treatment with the hypoxia mimetic $NiCl_2$, Plk3 phosphorylated the PTEN tumor-suppressor protein on Thr-366 and Ser-370 facilitating its stabilization and thereby increasing its overall activity[116] (Table 2). The results demonstrated that Plk3 is a player in the regulation of the PI3K/PDK1/Akt signaling during normoxic and hypoxic conditions by phosphorylation and

stabilization of PTEN, a negative regulator of the PI3K/PDK1/Akt pathway[121] (Figure 3d).

Hypoxia/reoxygenation stress-induced activation of Plk3 and c-Jun phosphorylation on Ser-63 and Ser-73 by Plk3 in human corneal epithelial cells leads to an increased DNA-binding activity of c-Jun and AP-1 resulting in increasing cell apoptosis[85] (Figure 3d; Table 2). In addition, immunofluorescence experiments demonstrated the co-localization of Plk3 and phospho-c-Jun in the nuclear region of hypoxia-induced cells.[85] Hypoxic stress-mediated activation of Plk3 results in growth attenuation and delay of corneal epithelial wound healing suggesting an important role of Plk3 in the hypoxia-induced signaling pathway[86] (Figure 3d).

Hyperosmotic stress

In response to extracellular hyperosmotic stress Plk3 is activated in human corneal epithelial cells and phosphorylates the transcription factors c-Jun on Ser-63 and Ser-73 and ATF-2 on Thr-71 resulting in the activation of the transcriptional factors independent from the activation of the JUN N-terminal kinase (JNK) and p38 signaling pathway[122,123] (Figure 3e; Table 2). In addition, immunofluorescence experiments demonstrated the co-localization of Plk3 and ATF-2 in the nuclear region of hyperosmotic stress-induced human corneal epithelial cells.[123] These results suggest that Plk3 functionally regulates the AP-1 transcriptional complex by direct phosphorylation of its components, ATF-2 and c-Jun, in response to hyperosmotic stress in parallel to JNK and p38 signaling pathway.

Endoplasmic reticulum stress

Endoplasmic reticulum (ER) stress, which results from protein misfolding within the secretory pathway, has a profound effect on cancer cell proliferation and survival. Following ER stress induced by tunicamycin, nuclear receptor liver receptor homolog-1 induces transcription of Plk3, which phosphorylates and activates the transcription factor ATF-2[124] (Figure 3f; Table 2). Plk3 inhibition results in decreased ability to resolve ER stress indicating that Plk3 is required for ER stress resolution.[124]

PLK3 EXPRESSION IN MALIGNANT TISSUES

In a broad spectrum of human tumors a gradual upregulation of Plk1 expression was observed from normal tissue to malignant tissue.[15,20,27,125,126] The level of Plk1 transcripts or protein directly correlates with patient prognosis in certain types of human cancer indicating that high Plk1 activity contributes to the aggressiveness of tumors. Far less is known about the contribution of the other family members to the progression of cancer.

Plk3-deficient mice are viable, although it is not clear whether they have a higher tumor incidence compared to the wild-type counterparts.[76,103] The expression of Plk3 was found to be reduced in certain types of human cancer like head and neck, lung and liver[39,50,58] (Table 3). Interestingly, a comprehensive study on Plk1–4 in HCC as assessed by means of real-time RT–PCR and western blot analysis observed the lowest level of Plk3 in HCC patients with poor survival (HCCP)[58] (Table 3). Although no promoter methylation was detected in normal livers, the Plk3 gene was silenced by promoter hypermethylation in 37.3% of HCC (Figure 1b). The degree of Plk3 promoter methylation correlated inversely with the survival of HCC patients. The analysis of the genomic status of Plk3 revealed loss of heterozygosity in 24% Plk3 gene loci. Moreover, loss of heterozygosity showed a significant correlation to promoter hypermethylation suggesting the inactivation of both alleles in these cases. The depletion of Plk3 in HCC cell lines induced accelerated growth. The evidence for describing the somatic inactivation by genetic and/or epigenetic mechanisms in human cancers establish a tumor-suppressor function for Plk3, which is supported by the localization of the human Plk3 gene to the short arm of chromosome 1 (1p34), a region that displays loss of heterozygosity or homozygous deletions in several types of cancers and which has been proposed to harbor tumor susceptibility genes.[39,127–129]

Remarkably, while the expression of Plk1 and Plk3 determined immunohistochemically was found to be low in normal ovarian surface epithelium and borderline tumors, in ovarian carcinomas, 26% of cases were Plk1 positive and 50.6% of cases were Plk3 positive (Table 3). The overexpression of Plk3 had an impact on patient prognosis with shortened survival time for patients with Plk3 ($P = 0.02$).[130] Moreover, in an immunohistochemical study of 135 breast carcinomas, overexpression was observed in 42.2% for Plk1 and 47.4% for Plk3 in breast carcinomas when compared with non-transformed breast tissue[131] (Table 3). Overexpression of Plk3 correlated significantly to reduce the median overall ($P < 0.001$) and relapse-free ($P = 0.021$) survival times in a multivariate survival analysis.

Taken together, while the expression of Plk3 is reduced in cancers of head/neck, lung and liver,[39,50,58] it is overexpressed in ovarian and breast cancer. A bad prognosis correlates with the downregulation of Plk3 in patients suffering from HCC. In contrast, bad prognosis is linked with overexpression of Plk3 in breast and ovarian cancer indicating apparently that a tumor-suppressor function for Plk3 cannot be generalized, but depends on the individual tumor type.

CONCLUDING REMARKS

The role of Plk3 in regulating cellular proliferation and apoptosis has been established since its early functional analysis in mammalian cells. Surprisingly, despite many fascinating functional traits its functional exploration has lagged behind that of Plk1, which is a validated target for fighting cancer cell proliferation. Various lines of evidence highlight the pleiotropic role of Plk3 in response to different types of cellular stresses (Figure 4). The stimulation of Plk3 as executioner of p53 safety functions may slow or even stop mitotic progression, giving normal cells a

Table 3. Deregulation of Plk3 expression in human cancer

Tumor type	*Experimental method*	*Number of patient samples*	*Plk3 expression*	*Plk3 expression correlates with prognosis and overall survival*	*References*
Lung	Northern blot	18	↓	×	39
Head and neck	Northern blot	35	↓ (74%)	×	28,50
Liver	qPCR	HCCB: 35 HCCP: 40	↓	✓	58
Ovary	IHC	77	↑ (50.6%)	✓	130
Breast	IHC	135	↑ (47.4%)	✓	131

Abbreviations: HCC, hepatocellular carcinoma; HCCB, HCC with better outcome; HCCP, HCC with poor survival; IHC, immunohistochemistry; Plk3, polo-like kinase 3.

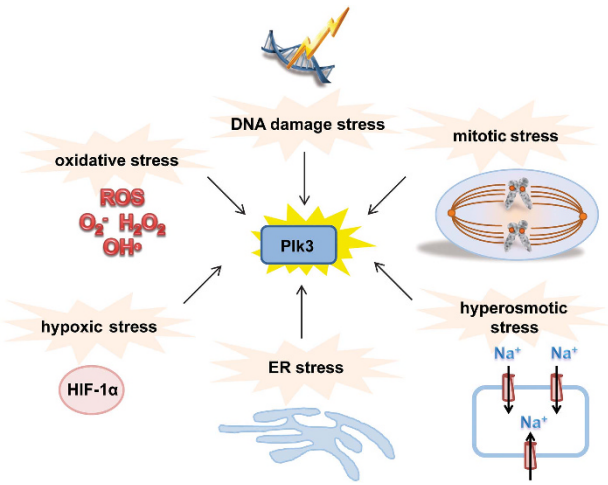


Figure 4. Plk3 is a stress response protein and is activated under various stress conditions, such as DNA damage, oxidative, mitotic, hypoxic and hyperosmotic stress. Furthermore, ER (endoplasmic reticulum) stress induces the transcription of Plk3.

chance to compensate for stressful situations or to prevent further stress overload. Considering the overarching importance of p53 for human cancer, it will be of the utmost importance to focus on the activities of all the Plk family members including Plk3. This will improve our understanding of the mutual regulatory network of p53 and the Plks in cancer. However, despite the immense significance of Plk3 for cancer cell signaling several hurdles need to be considered before we can improve the understanding of Plk3 functions: studies on the turnover rate of a large spectrum of mRNAs revealed that within the Plk family (Plk1–4) Plk3 mRNA has the highest turnover rate and is therefore more resistant to RNAi-mediated silencing compared to the other family members.[132] Therefore, a complete knockdown of Plk3 mRNA, which is required to clearly define its function in cancer cells, is very hard to reach. More sophisticated techniques like the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) system or homologues recombination might be helpful to eliminate the function of Plk3 in cancer cells completely. In addition, the discussion on the solubility of the Plk3 protein and the suitability of commercially available antibodies increase the complexity in functional studies of Plk3. The analysis of potential isoforms of Plk3 and the determination of the Plk3 interactome will be helpful to complete the picture of Plk3 signaling.

Since Plk3 is also targeted by ATP-competitive Plk1 inhibitors, its biological role in cancer cells requires careful analysis. Considering, in general, the evolutionary conservation of the ATP-binding pocket in protein kinases and in particular its conservation in Plks it is not surprising that all ATP-competitive inhibitors of Plk1 are also efficient in inhibiting Plk3. The simultaneous inhibition of Plk1 and Plk3 compared to the sole inhibition of Plk1 could be disadvantageous for the quality of new cancer drugs in regard to the function of Plk3 as tumor-suppressor gene and its function in stress response (Figure 4). However, Plks contain two important functional domains: the kinase domain and the PBD that regulates the function of Plks at different levels.[133] Recently, a new class of Plk inhibitors has been developed that target the PBD.[134] The comparison of the PBDs of different Plks has shown that the PBD is much less conserved compared to the ATP-binding site. However, a recent structural analysis has revealed that the core region of the binding cleft in the apo PBD (Plk2) is highly similar to that of PBD (Plk1) in complex with the phosphopeptide.[135] It will be interesting to explore whether the binding cleft within the PBD of Plk3 is similar to the corresponding binding groove of Plk1. An increasing number of studies provided convincing evidence that in addition to the kinase domain, the PBD is an attractive target for anticancer drug development. An early study revealed that interfering with the function of the PBD of Plk1 by treating cells with an Antennapedia peptide fused to the polo-box (amino acids 410–429) induces mitotic arrest followed by apoptosis.[136] Recent investigations extended the spectrum of functional PBD inhibitors to small molecules and peptide-related inhibitors.[137–141] This new class of inhibitors are likely to exclusively inhibit Plk1 without targeting the tumor-suppressor Plk3. Despite extensive efforts for the exploration of Plks in cell signaling, much needs to be learned to obtain a clear picture of Plk3's role in cancer cells and to improve treatment strategies against cancer using Plk-targeting drugs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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