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Protein-protein interactions of ASPP2: an emerging therapeutic target

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Abstract

The pro-apoptotic ASPP2 protein has a central role in regulating apoptosis in both p53-dependent and p53-independent pathways. It has become clear in recent years that ASPP2 has also a role in other important cellular processes like senescence and regulating cell polarity. ASPP2 interacts with numerous proteins in order to exert its pro-apoptotic effect while some other proteins inhibit its pro-apoptotic activity. These interactions are emerging potential targets for activation or inhibition. Drugs that activate ASPP2 may not only induce apoptosis of cancer cells, but also affect other disease-related cellular pathways. Here we review the interactions of ASPP2 with its partner proteins and their potential targeting for drug development.

Introduction

Apoptosis, programmed cell death, is one of the most important regulatory processes in the cell. Apoptosis maintains the tissues healthy and defends them from malignant transformation[1]. The ASPP (apoptosis stimulating proteins of p53) protein family has a key role in regulating apoptosis. ASPP2 and ASPP1 activate the p53-mediated apoptotic response, while iASPP inhibits it. The ASPP proteins are also involved in other apoptosis-related pathways and integrate many factors related to cell proliferation and apoptosis into the cellular decision-making process [2]. Frequently, iASPP is upregulated in cancer, while ASPP2 and ASPP1 are downregulated. This makes the ASPP proteins important emerging targets for developing anti-cancer lead compounds. In this review we will focus on ASPP2, the major and most characterized pro-apoptotic member of the ASPP protein family.

ASPP2 was originally identified as p53 binding protein 2 (53BP2), a 528 residues protein that bound wild type (WT) p53 core domain (CD) but not mutant p53[3]. A second 1005 residues isoform, termed Bbp, was discovered as a cytoplasmic protein that bound Bcl-2 and p53, but was unable to bind both of them simultaneously. Bbp induced apoptosis and was present in low levels in many normal human cells. Over-expression of p53 did not change the location of Bbp and it remained in the cytoplasm[4–7]. Bbp also induced apoptosis via the mitochondrial death pathway[8,9]. Over-expression of Bbp in cells resulted in an increased number of cells at the G2/M phase[4]. Later it was shown that both 53BP2 and Bbp are isoforms of the C-terminal part of a larger protein termed ASPP2 (Figure 1)[2]. Both Bbp and ASPP2 are splice variants of the gene TP53BP2, which is mapped to the long arm of chromosome 1 at q42.1[5,9]. ASPP2 induced the pro-apoptotic activity of p53 better then Bbp but did not induce the cell-cycle arrest activity of p53[2]. The full length 1128 residues ASPP2 is mostly cytoplasmic and is also involved in the mitochondrial death pathway[2,8]. While the full length ASPP2 and Bbp as well as the Bbp N-terminal domain (residues 1-758) are cytoplasmic, the C-terminal domains of ASPP2 and Bbp (Bbp 759-1005 and ASPP2 600-1128) can also be localized to the nucleus[2,7]. Since ASPP2 is mostly cytoplasmic, it is unclear how it induces the transcriptional activity of nuclear proteins like p53. In cerebral ischemia, which results in death of brain cells, ASPP2 levels in brain cells increase[10].

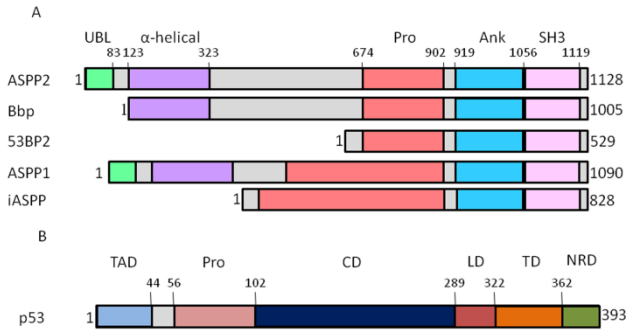

Figure 1: Domain organization of the ASPP proteins. (A) The ASPP2 domains include: an N-terminal domain with a β-Grasp ubiquitin-like fold (UBL), a putative α-helical domain, a Proline rich domain (Pro), Ankyrin repeats (Ank) and a src homology 3 domain (SH3); (B) p53 domains include: Transcription activation domain (TAD), Proline rich domain (Pro), core domain (CD), linker region (LD), tetramerization domain (TD) and the C-terminal negative regulatory domain (NRD).

ASPP2 induces the pro-apoptotic activity of p53

p53 is a transcription factor that induces apoptosis or cell cycle arrest in response to oncogenic stress such as DNA damage. Thus, p53 protects the cell from malignant transformation and it is not surprising that the TP53 gene is mutated in about 50% of human cancers [11–14]. ASPP2 interacts with p53 *in vivo* and, in response to apoptotic stimuli, induces its pro-apoptotic activity by inducing the binding of p53 to its target DNA followed by the transactivation of pro-apoptotic genes. Among the p53 target genes, upregulated by ASPP2 are PIG3, PUMA and Fas/CD95 but not Mdm2 and cyclin G. The effect of ASPP2 on the p53 targets p21 and Bax is not conclusive [2,5,15,16]. ASPP2 does not induce the cell cycle arrest activity of p53[2,5,17]. Endogenous ASPP2 interacts with endogenous p53 and the complex formation is UV irradiation dependent[2]. In breast carcinomas, ASPP2 is frequently downregulated in WT p53 tumors, but not in mutant p53 tumors[2]. Drosophila ASPP does not activate drosophila p53 dependent apoptosis, suggesting that the regulation of apoptosis by ASPP developed later in evolution[18]**.** ASPP2 requires both p53CD and the p53 transactivation activity in order to induce p53 dependent apoptosis[19].

ASPP2 and cancer

ASPP2 is frequently downregulated in human cancer, regardless to the p53 status[2,20–27]. It was suggested that ASPP2 levels are low due to hypermethylation of the ASPP2 gene promoter or of the promoter regulatory sites[20,22]. One functional copy of the ASPP2 gene is insufficient for the tumor suppressor activity of WT ASPP2: ASPP2 +/- mice develop large amount of tumors , specifically high-grade Lymphomas and rhabdomyosarcomas, compared to ASPP2 +/+ mice as a response to γ-irradiation[28–30]. ASPP2 null mice show an early

embryonic death and the few who survive birth die after up to 30 days[28,29]. In mice, ASPP2 depresses sarcoma tumors regardless of the p53 status (WT or mutant)[29]. In response to γ-irradiation, ASPP2 +/- primary thymocytes (hematopoietic progenitor cells present in the thymus) showed low levels of apoptosis and ASPP2 +/- mouse embryonic fibroblasts (MEFs) showed defective G0/G1 cell cycle checkpoint and completely different gene expression pattern in comparison to ASPP2 +/+ MEFs[28,31]. Bbp and ASPP2 inhibited the transformation of fibroblasts transfected with the oncogenes RAS and E1F[5,32]. In hepatocellular carcinoma cells and nude mice, shRNA against ASPP2 led to cancer cells growth and inhibition of apoptosis due to serum starvation[20]. Reduced ASPP2 expression is tightly associated with poor prognosis of cancer patients[25,33]. Cancer cell lines that express higher levels of ASPP2 mRNA are more sensitive to various DNA-damaging agents like UV, X-ray, doxorubicin, and cisplatine than cell lines expressing lower levels of ASPP2 mRNA, which show resistance to some chemotherapy agents[6,25,34]. In carcinoma, ASPP2 inhibited cell migration by Csk dependent inactivation of Src protein[26]. As inactivation of ASPP2 is strongly linked to cancer development, ASPP2 can serve has a good target for developing anti-cancer drugs that will activate it or mimic its activity.

Regulation of ASPP2 levels

Not much is known about the regulation of ASPP2. It is clear, however, that the regulation of ASPP2 expression is complex and involves both p53-dependent and p53-independent mechanisms. In healthy cells p53 suppresses 53BP2 levels, but upon DNA damage 53BP2 and ASPP2 levels increase resulting in activation of DNA damage-induced apoptosis[35,36]. Mdm2 and Mdmx inhibit the transcriptional activity of p53 and by doing so inhibit ASPP2 ability to induce the apoptotic activity of p53 [19]. One of the regulators of ASPP2 expression is E2F, a known activator of p53 apoptotic activity. E2F binds *in vivo* to the ASPP2 promoter and upregulates ASPP2 levels [37–39]. Treatment of cells with proteasome inhibitors revealed that proteasomal degradation modulates the ASPP2 protein levels and apoptotic function[40].

The structure of ASPP2

ASPP2 is composed of structured and disordered domains. The N-terminal part (residues 1-83) has a β-Grasp ubiquitin-like fold (Figure 2A)[41]. This is followed by a predicted α-helical domain between residues123-323[4]. The ASPP2 C-terminal part contains a disordered Proline-rich (Pro) domain followed by four Ankyrin repeats and an SH3 domain (Ank-SH3) [3,4,42]. The crystal structure of the ASPP2 Ank-SH3 domains (residues 926-1118) was first solved in

complex with p53CD (residues 97-287) (Figure 2B) [43]. The Ank-SH3 domains also mediate the interactions of ASPP2 with other partner proteins such as Bcl-2 and NFkB (see below)[3,44]. The Ank repeats can also serve as a nuclear import sequence[45]. ASPP2 Pro is intrinsically disordered[42].

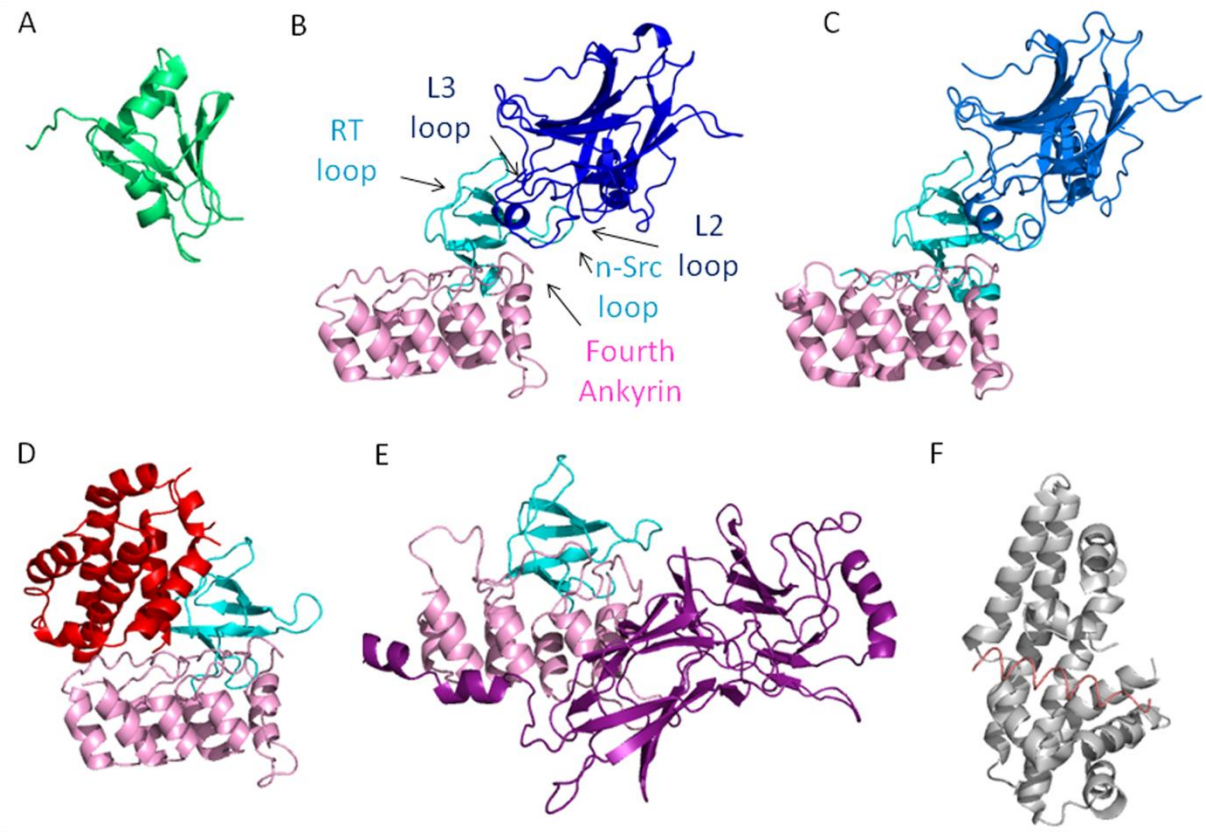

Figure 2: Structure of ASPP2. (A) The MR structure of the ASPP2 N-terminus (residues 1-83, Green) shows a β-Grasp ubiquitin-like fold[41] (PDB ID:2UWQ); (B-E) ASPP2 C-terminal Ankyrin repeats (Pink) and SH3 domain (Cyan) in complex with: (B) p53CD (Blue), crystal structure[43]; PDB ID: 1YCS; (C) p73 CD (Blue), crystal structure[58]; PDB ID:4A63; (D) Bcl-2 (Red), model[69]; (E) NFκB p65 (Purple), model[72];(F) CagA 23-221 (Grey) bound to ASPP2 746-765 derived peptide (Orange); PDB ID: 4IRV[47]. The figure was made using Pymol[96].

An intramolecular autoinhibitory interaction between the Ank-SH3 and Pro domains of ASPP2: Pulldown assays showed that ASPP2 Ank-SH3 and ASPP2 Pro interact with each other intramolecularly *in vitro*. Using peptide array screening and fluorescence anisotropy the specific binding sites between these domains were revealed. ASPP2 Pro residues 693-712 and 723-737 bound ASPP2 Ank-SH3 residues 931-961 and 1083-1096[42]. The intramolecular domain-domain interaction regulates the intermolecular interactions of ASPP2 by an auto-inhibitory mechanism (Figure 3)[42,46]. In cells, a peptide derived from ASPP2 Pro 726-782 disrupted the interaction between ASPP2 and p53, possibly because of this regulatory

mechanism[47]. Competition experiments showed that ASPP2 Pro and the ASPP2-binding proteins p53, Bcl-2 and NFkB compete for the same binding site in ASPP2 Ank-SH3. ASPP2 Pro 723-737 and p53CD displaced NFκB 303-332 from binding ASPP2 Ank-SH3. p53CD displaced ASPP2 Pro 723-737 from binding ASPP Ank-SH3 and its derived peptide 1083-1096[42,46]. The binding sites of ASPP2 to p53 CD, Bcl-2, and NFkB are different, yet lie on the same face of ASPP2 Ank-SH3 (Figure 4). The intramolecular binding site to the Pro domain overlaps these three intermolecular binding sites, in support of a regulatory role of this intramolecular interaction[46,48].

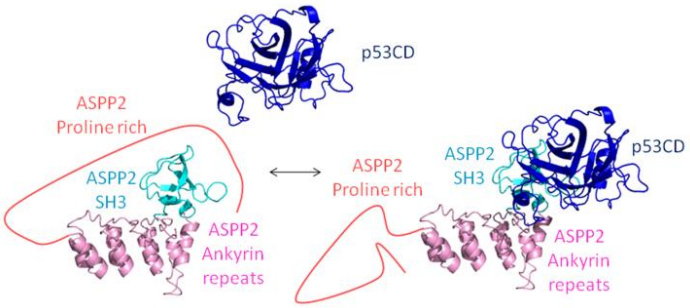

Figure 3: An intramolecular interaction in ASPP2 regulates its protein-protein interaction. The intramolecular interaction between ASPP2 Pro (Orange) and ASPP2 Ank-SH3 (Pink and Cyan) regulates the interaction of ASPP2 Ank-SH3 with p53CD (Blue); PDB ID: 1YCS[43]. The figure was made using Pymol[96].

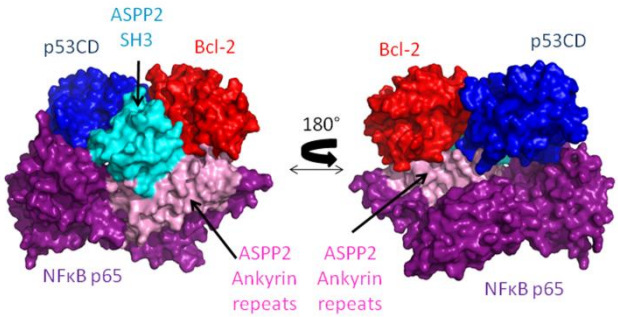

Figure 4: The binding sites of ASPP2 (Pink and Cyan) to p53 CD (Blue), Bcl-2 (Red) and NFkB (Purple) are different, yet lie on the same face of ASPP2 Ank-SH3; PDB ID: 1YCS[43] and models[69,72]. The figure was made using Pymol[96].

Protein-protein interactions of ASPP2

ASPP2 interacts with numerous protein partners, which are all involved in important regulatory processes in the cell such as apoptosis. These interactions can result in different effects (Figure 5). Many of these interactions are potential targets for inhibition and are emerging targets for anti-cancer drug design. Below we provide details of the current knowledge about these interactions.

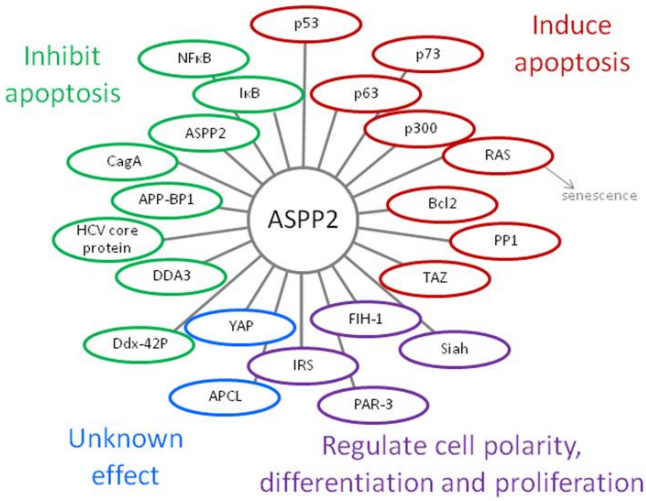

Figure 5: The interaction network of ASPP2: ASPP2 binds numerous proteins that are involved in many cellular pathways. These interactions can be targets for therapeutic intervention. See text for details.

1. The ASPP2 – p53 interaction

ASPP2 binds the Pro domain, core domain and linker region of p53 (Figure 1B)[49,50]. p53 cannot bind its DNA targets and ASPP2 Ank-SH3 simultaneously since the binding sites for the DNA and ASPP2 in p53CD overlap[3,51,52]. The n-Src loop (residues 1089-1097) and RT loop (residues 1068-1076) of the ASPP2 SH3 domain bind the L3 loop (residues 236-251) of p53 while the fourth Ank repeat (residues 1021-1027) of ASPP2 binds the L2 loop of p53 (residues 163-195)[43]. These sites in p53 are frequently mutated in cancer[43].The affinity of ASPP2 Ank-SH3 to the different p53 domains was extensively studied and the results are summarized in table 1. Two different K_d values were measured for the p53CD– ASPP2 Ank-SH3 interaction, which were either tens of nanomolars or micromolars (Table 1). The differences may be explained by the different techniques used: In ELISA and SPR one of the binding partners is attached to a solid surface, while in ITC both binding partners are in solution. The differences may also be explained by the use of slightly different proteins fragments in each affinity measurement. The different techniques and protein fragments used are detailed in table 1. The ASPP2 1089-1097 peptide, derived from the n-src loop, had a high affinity to p53CD. This peptide stabilized and reactivated p53 mutants *in vitro* and in cells[53,54]. The ASPP2 interaction with p53 is regulated by the intramolecular interaction between the ASPP2 Ank-SH3 and ASPP2 Pro domains[42,46]. Inducing p53-dependent apoptosis by stimulating ASPP2 in cancer cells is an attractive approach for designing anti-cancer drugs[2].

Table 1: Binding of the Ank-SH3 domains of ASPP2 to p53

	p53 Core domain				p53 Proline rich+ Core domains		p53 Linker	p53 Proline rich + Core + linker + tetramerization + basic domains
p53 residues	94-312	94-312	94–292	94-292	56–289	56–289	289–322	56-393
ASPP2 residues	891-1128	902-1128	905-1128	890-1128	925–1128	925–1128	925–1128	925-1128
Method	ITC	ITC	ELISA	SPR	ITC	NMR	NMR	Fluorescence anisotropy
K_d (μM)	2.2±0.2[51]	5[52]	0.023±0.002[16]	0.03[43]	1.5±0.1[49]	1.3±0.2[49]	40±5[49]	1.9±0.3[49]

2. ASPP2 interactions with p63 and p73

The p53 protein family members p63 and p73 are transcription factors that regulate cell cycle arrest and apoptosis. Both are important for proper cell development and have only a minor role in tumor suppression[55,56]. ASPP2 interacts with the p63 and p73 *in vivo* and *in vitro*[52,57].Using RNAi against p63 or p73 revealed that most of the p53-independent ASPP2-induced apoptosis is performed by inducing the apoptotic activity of p63 and p73 in response to DNA damage or Cisplatin. ASPP2 induces apoptosis through p63 and p73 by inducing the expression of Bax, PIG3 and PUMA but not mdm2 and p21$^{WAF-1/CIP1}$, apparently by transactivating these genes[57]. In Squamous cell carcinoma, ASPP2 represses the expression of p63, by inducing NFκB activity. Downregulation of ASPP2 in these cells, which express an oncogenic isoform of p63, resulted in tumor metastasis[27]. p63 and p73 are unable to bind their target DNA and ASPP2 simultaneously[52]. The crystal structure of ASPP2 Ank-SH3 in complex with p73CD shows that the p73CD binding interface to ASPP2 is very similar to that of p53CD, but the binding interface between ASPP2 and p73 is smaller than the interface between ASPP2 and p53 (Figure 2C)[58]. A model of ASPP2 Ank-SH3 in complex with p63CD (residues 154–365) was made based on the crystal structure of the complex between p53 and ASPP2. The binding affinities of ASPP2 Ank-SH3 to p63CD and p73CD range from tens of nanomolar to few micromolar[52,59]. The different affinities measured and the techniques and proteins fragments that were used are described in table 2. Native gel mobility shift assay showed that the affinity of ASPP2 to p53CD and p73CD is at the low micromolar range[58].

Table 2: Binding of ASPP2 Ank-SH3 to the p53 protein family members

	p53 Core domain		p63 Core domain		p73 Core domain	
p53CD family residues	p53 94-312	p53 94-292	p63 154-365	p63 123-323	p73 104-333	p73 112-312
ASPP2 residues	902-1128	905-1128	902-1128	905-1128	902-1128	905-1128
method	ITC	ELISA	ITC	ELISA	ITC	ELISA
K_d (μM)	5[52]	0.023±0.002[59]	2.5[52]	0.34±0.04[59]	2.1[52]	1.0±0.2[59]

3. ASPP2 interaction with Ras

The Ras oncoprotein regulates the cell cycle, apoptosis and senescence and by that affects cell proliferation and morphology[60,61]. ASPP2 interacts with Ras and this interaction regulates the activity of both proteins. The sequence of ASPP2 1-83 has some similarity to sequences of other Ras binding or Ras associating domains present in proteins like c-Raf and PI3K although some important positive residues are not conserved[41]. ASPP2 induces activation of Ras. The N-terminal domain of ASPP2 (residues 1-123) interacts with Ras resulting in induction of p53 dependent apoptosis. This is possibly mediated by relocation of the complex from the cell membrane to the cytosol and nucleus[62,63]. Ras induces the activity of MAP kinase that phosphorylates ASPP2 on Ser 826. This Phosphorylation is required for the apoptotic activity of the ASPP2-Ras, complex. Thus Ras creates a feedback loop for amplification of the ASPP2-dependent apoptotic response[64]. ASPP2 also induces Ras dependent senescence, which is p53-independent[63,65,66]. Quantitative biophysical studies showed using NMR and ITC that the ASPP2 N terminal domain (residues 1-83) does not bind directly to H-Ras[41]. It is possible that the interaction in cells requires other factors or post-translational modifications.

4. **ASPP2 interactions with the Bcl-2 family proteins:** The Bcl-2 protein family contains both pro-apoptotic and anti-apoptotic members and has an important role in regulating apoptosis. Bcl-2, Bcl-X and Bcl-w are among the anti-apoptotic family members[67]. ASPP2 is known to interact with Bcl-2 since the discovery of the Bbp isoform (see above). In the cytoplasm, Bbp co-localizes with Bcl-2[4]. Overexpression of Bcl-2 and Bcl-x inhibited the mitochondrial apoptotic activity of Bbp in cells[68]. The Bcl-2- ASPP2 interaction requires both the Ank and SH3 domains of ASPP2[4]. Peptide array screening and biophysical methods showed that ASPP2 Ank-SH3 binds the three Bcl-2 family members Bcl-2, Bcl-x and Bcl-w.

The major binding site for ASPP2 is the BH4 domain of the Bcl-2 family proteins (Bcl-2 7-24, Bcl-x 6-26 and Bcl-w 6-28) that is important for their anti-apoptotic activity. ASPP2 also binds a second site in the Bcl-2 proteins, which serves as a binding site for the pro-apoptotic proteins from the Bcl-2 family (Bcl-2 89-120, Bcl-x 103-123, Bcl-w 41-67). This suggests that ASPP2 induces apoptosis by binding the Bcl-2 family anti-apoptotic proteins and releasing the Bcl-2 pro-apoptotic proteins from the complex with them. Among the Bcl-2 family members, ASPP2 had the highest affinity to peptides derived from Bcl-2, due to a larger proportion of positively charged residues in these peptides compared to peptides from the other Bcl-2 proteins. A computational model for the complex between ASPP2 Ank-SH3 and Bcl-2 was also made (Figure 2D)[69].

5. ASPP2 interaction with NFkB: NFκB is a key transcription factor involved in regulating the immune response and apoptosis[70]. Bbp binds the NFκB p65 subunit *in vivo* and *in vitro*. NFκB activation inhibits the apoptotic activity of Bbp in cells[68]. Co-transfection of HeLa cells with Bbp and p65 resulted in inhibition of Bbp-induced apoptosis, although the Bbp protein level in the cell did not change[7]. ASPP2 activates NFκB, resulting in inhibition of the neddylation pathway and specifically inhibition of APP-BP1 induced proliferation of dividing cells and inhibition of APP-BP1 induced apoptosis of neurons[71]. ASPP2 binds p65 through its Ank and SH3 domains but not the Pro region[7,42]. ASPP2 Ank-SH3 binds p65 derived peptides corresponding to residues 21-50 and 303-355 and the affinity to p65 303-332 was shown to be 0.27 μM[42]. A computational model of the complex between ASPP2 Ank-SH3 and p65 shows that ASPP2 binds residues 236-253 and 293-313 of p65, which also mediate the binding of NFκB p65 to its natural inhibitor IκB (Figure 2E)[72]. This similarity suggests that ASPP2 may induce NFκB p65 activation by displacing its natural inhibitor IκB**.**

6. **ASPP2 interactin with the *Helicobacter pylori* cytotoxin associated antigen A (CagA):** *H. pylori* infection is the strongest known risk factor for gastric cancer. CagA is one of the most important factors that link infection with *H.Pylori* to the development of gastric cancer[73,74]. The gene TP53BP2 that expresses ASPP2 was upregulated in *H.pylori*-exposed gastric epithelial cells[75]. Upon infection of cells with *H.pylori*, endogenous ASPP2 relocates near the bacteria attachment[47]. ASPP2 330-861 interacts with *H. Pylori* CagA 1-877 in cells and this interaction is followed by relocation of ASPP2 near the cell membrane[76]. Yeast two-hybrid experiments with the CagA N-terminus (19-257) as bait showed that it binds the ASPP2 Pro (residues 684-891) and more specifically residues 726-782 of ASPP2[47].

Following its interaction with CagA and only in the presence of CagA, ASPP2 recruits p53 but instead of promoting apoptosis, ASPP2 interaction with p53 and CagA results in inhibition of apoptosis. This interaction results in proteasomal degradation of p53 and consequently inhibition of the apoptotic response of the host cell. A tertiary complex between the three proteins was not observed. Cells transfected with *H.pylori* showed resistance to apoptosis as a response to the apoptosis inducing agent Doxorubicin. However, *H.pylori*-infected cells that where treated with ASPP2 shRNA were not resistant to Doxorubicin-induced apoptosis[76]. ASPP2 726-782 and ASPP2 746-765 interact with CagA in cells and disrupt CagA interaction with endogenous ASPP2, resulting in apoptosis of cells expressing ASPP2 726-782. ASPP2 726-782 disrupts the interaction between ASPP2 and p53, but it is possible that this is due to ASPP2 regulatory intramolecular interaction, that includes the peptide region and not the disruption of ASPP2-CagA interaction[42,46,47]. The crystal structure of CagA N-terminus 19-257 complex with the ASPP2 derived peptide 726-782 was solved, showing that CagA forms a deep cleft in which the ASPP2 peptide binds and forms a helix (Figure 2F). Most mutations performed in the binding residues of both proteins based on the crystal structure did not disrupt the interaction between the proteins *in vivo* and *in vitro*. The mutations that did have an inhibitory effect are ASPP2 726-782 Y754A or K751A and CagA I105A. These residues are thus the most important residues for the interaction. Only the ASPP2 Y754A mutation completely abolished the interaction between the proteins. Multiple mutations in CagA 19-235 were required for disrupting the complex (CagA 19-235 F114A+W212A, I105A+V107A, and F114A+F219A)[47].

7. **Protein phosphatase 1 (PP1):** ASPP2 interacts with the C-terminal part of PP1 (residues 896-1030) *in vitro* and *in vivo*[77,78]. ASPP2 inhibits PP1 activity specifically towards certain substrates such as glycogen phosphorylase but not myosin p-light chain[77,79]. ASPP2 is unable to bind PP1 and p53 simultaneously[77]. The Ank repeats of ASPP2 , between residues 903-934, interact with the consensus binding motif RVKF of PP1[77–79]. A peptide derived from ASPP2 903-934 inhibited PP1 binding to proteins that regulate PP1 activity, M_{110} and G_L, which also contain the RVKF motif[79]. A computational model that was confirmed by binding experiments with mutated proteins, predicted that PP1 260-261, which is positively charged, interacts with Glu 938 and ASP 940 of ASPP2. A peptide derived from PP1301-330 includes a Proline rich motif PXXPXR, a consensus sequence that binds SH3 domains. This peptide binds ASPP2 probably through it SH3 domain[80]. However deleting the SH3 domain

did not affect the interaction of ASPP2 and PP1[77]. Peptide array screening showed that ASPP2 Ank-SH3 binds PP1 residues 19-48 and 297-323[42].

8. Regulation of cell polarity– the ASPP2 interactions with PAR-3, FIH-1 and Siah2: The PAR complex regulates mammalian cell polarity[81]. Endogenous ASPP2 interacts with the PAR complex through it N-terminal residues (1-353) that bind the PAR-3 N and C termini (residues 1-269 and 584-1337). In cells, the ASPP2 - PAR-3 complex is important for epithelial cell polarization[82]. The complex has a role in regulating the formation of cell tight Junctions and in the development and maintenance of apical domains[82,83]. In mice, ASPP2 has an important role in regulating neuroepithelium polarity, differentiation, cells organization and tissue 3D morphology, and inhibits uncontrolled proliferation of progenitor cells[83]. ASPP2 co-localizes with PAR-3 and the PAR complex in apical cell-cell junction of polarized epithelial cell *in vivo* and in cells[82,83]. In polarizing cells, ASPP2 interacts with PAR-3 in the future location of the cell-cell interaction even before the PAR complex formation. ASPP2 and PAR-3 do not localize in this area unless they are in a complex [82,83]. The ASPP2-PAR-3 interaction is not affected by the PAR complex inhibitor LgI[82] but other factors regulate the activity of the ASPP2-PAR-3 complex: ASPP2 is hydroxylated *in vivo* on N983 by FIH-1. FIH-1 knockdown impaired ASPP2 interactions with PAR-3 and relocated them to the cytoplasm[84]. Under hypoxia, Siah2 interacts with ASPP2 resulting in proteasomal degradation of ASPP2 and downregulation of ASPP2 activity in regulating tight junction integrity, tissue morphology and cell polarity [85].

9. **Other protein partners of ASPP2**: ASPP2 was also shown to interact with other proteins including: Insulin receptor substrates[86–88], APP-BP1[71], Adenomatous polyposis coli protein-like (APCL)[89], Yes-associated protein (YAP) [42,90,91] ,p300[92], Hepatitis C virus (HCV) core protein[15,42,93], DDA3[94], TAZ[95], and DEAD box protein, Ddx42p[36]. Not much is known yet about the mechanism of these interactions and their biological effect.

Conclusions- ASPP2 as an anti-cancer drug target

ASPP2 is downregulated in many types of cancer cells. This results in impaired apoptosis of these cells and is one of the major reasons for their survival. ASPP2 carries out its function by binding to numerous proteins involved in many cellular pathways. Taken together, this sets the interactions of ASPP2 as emerging targets for the development of anti-cancer lead compounds (Figure 6).

The interactions of ASPP2 are involved in regulation of apoptosis both in the p53 dependent and independent pathways. The ASPP2 interactions with p53 family members are highly important in the context of the p53-dependent apoptosis. By mimicking or activating the ASPP2 activity, the p53 apoptotic activity can be induced. A peptide derived from the ASPP2 n-src loop (residues 1089-1097) made cancer cells expressing WT p53, but not p53 null cells, sensitive to γ -radiation[54]. Other important potential targets are the ASPP2 interactions with p63, p73, Bcl-2 and Ras, which are key proteins in apoptotic and oncogenic pathways. Drugs that will mimic ASPP2 will induce the pro-apoptotic activity of these proteins and will lead to apoptosis of cancer cells. Other potential targets are inhibition of the anti-apoptotic outcome of the ASPP2 interactions with CagA, NFκB and HCV core protein. Reversing the role of ASPP2 back to pro-apoptotic is the challenge in these cases.

There is a lot to reveal about the ASPP2 interactions network and this information is essential for target definition and for addressing specificity issues of potential ASPP2-inhibitory lead compounds. Taken together, the currently existing data shows that ASPP2 interactions are highly important emerging targets for developing anti-cancer lead compounds. Studying the exact molecular mechanisms of these interactions is essential for targeting ASPP2 for therapeutic purposes.

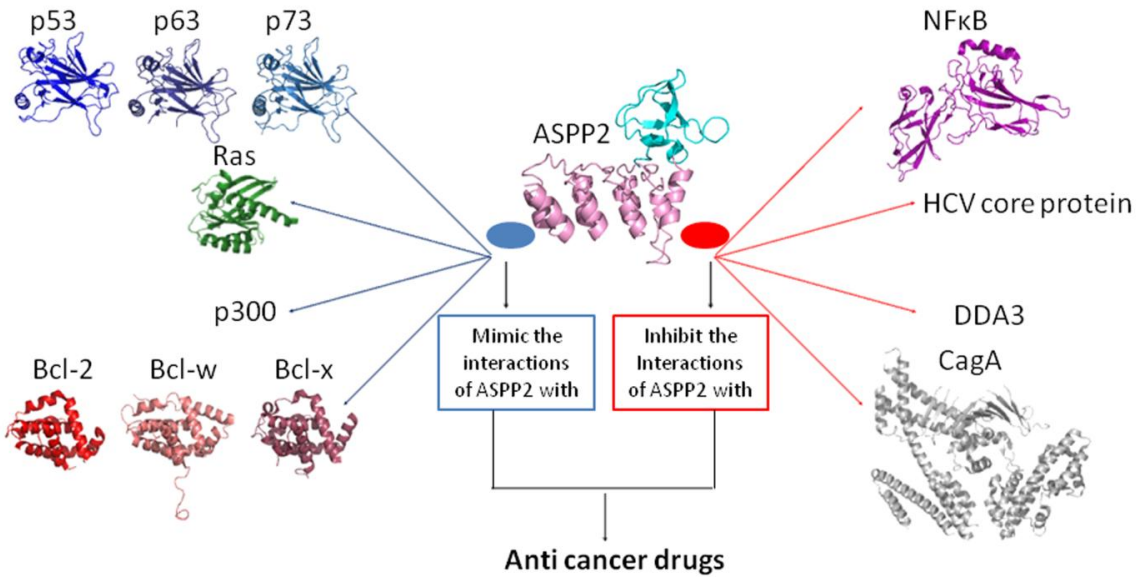

Figure 6: ASPP2 as an anti-cancer drug target: ASPP2 (Cyan and Pink, PDB ID:1YCS[43]) binds numerous protein partners that inhibit its apoptotic activity (Red: NFκB, Purple, PDB ID: 1LEI[97]; HCV core protein; DDA3; and CagA, Grey, PDB ID: 4DVY[98]) or induce it (blue: p53 protein family, shades of Blue, PDB IDs: 1YCS[43], 3QYN[99] and 4A63[58]; Ras, Green, PDB ID: 6Q21[100]; p300; and Bcl-2 anti-apoptotic family members, shades of Red, PDB IDs: 4IEH[101], 1O0L[102] and 3SP7.). The red and blue circles Represent small molecules that can serve as potential anti-cancer drugs by interfering with ASPP2 interactions. Note that the location of these drugs in the figure does not necessarily represent their actual binding site on the ASPP2 structure. The figure was made using Pymol[96].

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References:

1. S. Elmore, *Toxicol. Pathol.*, 2007, **35**, 495–516.

2. Y. Samuels-Lev, D. J. O'Connor, D. Bergamaschi, G. Trigiante, J. K. Hsieh, S. Zhong, I. Campargue, L. Naumovski, T. Crook, and X. Lu, *Mol. Cell*, 2001, **8**, 781–94.

3. K. Iwabuchi, P. L. Bartel, B. Li, R. Marraccino, and S. Fields, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 6098–102.

4. L. Naumovski and M. Cleary, *Mol. Cell. Biol.*, 1996, **16**, 3884–3892.

5. K. Iwabuchi, B. Li, H. F. Massa, B. J. Trask, T. Date, and S. Fields, *J. Biol. Chem.*, 1998, **273**, 26061–8.

6. Y. Ao, L. H. Rohde, and L. Naumovski, *Oncogene*, 2001, **20**, 2720–5.

7. J. P. Yang, M. Hori, N. Takahashi, T. Kawabe, H. Kato, and T. Okamoto, *Oncogene*, 1999, **18**, 5177–86.

8. S. Kobayashi, S. Kajino, N. Takahashi, S. Kanazawa, K. Imai, Y. Hibi, H. Ohara, M. Itoh, and T. Okamoto, *Genes Cells*, 2005, **10**, 253–60.

9. N. Takahashi, S. Kobayashi, X. Jiang, K. Kitagori, K. Imai, Y. Hibi, and T. Okamoto, *Biochem. Biophys. Res. Commun.*, 2004, **315**, 434–8.

10. K. Liu, H. Zhao, H. Yao, S. Lei, Z. Lei, T. Li, and H. Qi, *Biomed Res. Int.*, 2013, **2013**, 867537.

11. M. Oren, *Cell Death Differ.*, 2003, **10**, 431–42.

12. B. Vogelstein, D. Lane, and a J. Levine, *Nature*, 2000, **408**, 307–10.

13. K. H. Vousden and X. Lu, *Nat. Rev. Cancer*, 2002, **2**, 594–604.

14. T. Soussi, S. Kato, P. P. Levy, and C. Ishioka, *Hum. Mutat.*, 2005, **25**, 6–17.

15. Y. Cao, T. Hamada, and T. Matsui, *Biochem. Biophys. Res. Commun.*, 2004, **315**, 788–795.

16. A. M. Wilson, B. Morquette, M. Abdouh, N. Unsain, P. a Barker, E. Feinstein, G. Bernier, and A. Di Polo, *J. Neurosci.*, 2013, **33**, 2205–16.

17. S. K. Thukral, G. C. Blain, K. K. Chang, and S. Fields, *Mol. Cell. Biol.*, 1994, **14**, 8315–21.

18. P. F. Langton, J. Colombani, B. L. Aerne, and N. Tapon, *Dev. Cell*, 2007, **13**, 773–82.

19. D. Bergamaschi, Y. Samuels, S. Zhong, and X. Lu, *Oncogene*, 2005, **24**, 3836–41.

20. J. Zhao, G. Wu, F. Bu, B. Lu, A. Liang, L. Cao, X. Tong, X. Lu, M. Wu, and Y. Guo, *Hepatology*, 2010, **51**, 142–53.

21. W.-K. Liu, X.-Y. Jiang, J.-K. Ren, and Z.-X. Zhang, *Onkologie*, 2010, **33**, 500–3.

22. Z.-J. Liu, X. Lu, Y. Zhang, S. Zhong, S.-Z. Gu, X.-B. Zhang, X. Yang, and H.-M. Xin, *FEBS Lett.*, 2005, **579**, 1587–90.

23. Z.-J. Liu, Y. Zhang, X.-B. Zhang, and X. Yang, *Leukemia*, 2004, **18**, 880.

24. D. Sgroi, S. Teng, and G. Robinson, *Cancer Res.*, 1999, **59**, 5656–5661.

25. M. M. Schittenhelm, B. Illing, F. Ahmut, K. H. Rasp, G. Blumenstock, K. Döhner, C. D. Lopez, and K. M. Kampa-Schittenhelm, *PLoS One*, 2013, **8**, e80193.

26. V. C. Y. Mak, L. Lee, M. K. Y. Siu, O. G. W. Wong, X. Lu, H. Y. S. Ngan, E. S. Y. Wong, and A. N. Y. Cheung, *Carcinogenesis*, 2013, **34**, 2170–7.

27. L. Tordella, S. Koch, V. Salter, A. Pagotto, J. B. Doondeea, S. M. Feller, I. Ratnayaka, S. Zhong, R. D. Goldin, G. Lozano, F. D. McKeon, M. Tavassoli, F. Fritzsche, G. F. Huber, M. Rössle, H. Moch, and X. Lu, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 17969–74.

28. K. Kampa, J. Acoba, and D. Chen, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 4390–4395.

29. V. Vives, J. Su, S. Zhong, and I. Ratnayaka, *Genes Dev.*, 2006, **20**, 1262–1267.

30. V. Vives, E. Slee, and X. Lu, *Cell Cycle*, 2006, **5:19**, 2187–2190.

31. K. M. Kampa, M. Bonin, and C. D. Lopez, *Cell Cycle*, 2009, **8**, 2871–2876.

32. D. Bergamaschi, Y. Samuels, N. J. O'Neil, G. Trigiante, T. Crook, J.-K. Hsieh, D. J. O'Connor, S. Zhong, I. Campargue, M. L. Tomlinson, P. E. Kuwabara, and X. Lu, *Nat. Genet.*, 2003, **33**, 162–7.

33. I. S. Lossos, Y. Natkunam, R. Levy, and C. D. Lopez, *Leuk. Lymphoma*, 2002, **43**, 2309–17.

34. T. Mori, H. Okamoto, N. Takahashi, R. Ueda, and T. Okamoto, *FEBS Lett.*, 2000, **465**, 124–8.

35. C. D. Lopez, Y. Ao, L. H. Rohde, T. D. Perez, D. J. O'Connor, X. Lu, J. M. Ford, and L. Naumovski, *Mol. Cell. Biol.*, 2000, **20**, 8018–25.

36. H. Uhlmann-Schiffler, S. Kiermayer, and H. Stahl, *Oncogene*, 2009, **28**, 2065–73.

37. D. Chen, E. Padiernos, F. Ding, I. S. Lossos, and C. D. Lopez, *Cell Death Differ.*, 2005, **12**, 358–68.

38. V. Fogal, N. N. Kartasheva, G. Trigiante, S. Llanos, D. Yap, K. H. Vousden, and X. Lu, *Cell Death Differ.*, 2005, **12**, 369–76.

39. T. Hershko, M. Chaussepied, M. Oren, and D. Ginsberg, *Cell Death Differ.*, 2005, **12**, 377–83.

40. Z. Zhu, J. Ramos, K. Kampa, S. Adimoolam, M. Sirisawad, Z. Yu, D. Chen, L. Naumovski, and C. D. Lopez, *J. Biol. Chem.*, 2005, **280**, 34473–80.

41. H. Tidow, A. Andreeva, T. J. Rutherford, and A. R. Fersht, *J. Mol. Biol.*, 2007, **371**, 948–58.

42. S. Rotem, C. Katz, H. Benyamini, M. Lebendiker, D. Veprintsev, S. Rüdiger, T. Danieli, and A. Friedler, *J. Biol. Chem.*, 2008, **283**, 18990–9.

43. S. Gorina and N. P. Pavletich, *Science*, 1996, **274**, 1001–5.

44. Y. Cho, S. Gorina, P. Jeffrey, and N. Pavletich, *Science*, 1994, **265**, 346–355.

45. S. Sachdev, a Hoffmann, and M. Hannink, *Mol. Cell. Biol.*, 1998, **18**, 2524–34.

46. S. Rotem-Bamberger, C. Katz, and A. Friedler, *PLoS One*, 2013, **8**, e58470.

47. D. Nesic, L. Buti, X. Lu, and C. E. Stebbins, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, 1562–7.

48. H. Benyamini and A. Friedler, *J. Mol. Recognit.*, 2011, **24**, 266–74.

49. J. Ahn, I.-J. L. Byeon, C.-H. Byeon, and A. M. Gronenborn, *J. Biol. Chem.*, 2009, **284**, 13812–22.

50. D. Bergamaschi, Y. Samuels, A. Sullivan, M. Zvelebil, H. Breyssens, A. Bisso, G. Del Sal, N. Syed, P. Smith, M. Gasco, T. Crook, and X. Lu, *Nat. Genet.*, 2006, **38**, 1133–41.

51. H. Tidow, D. B. Veprintsev, S. M. V Freund, and A. R. Fersht, *J. Biol. Chem.*, 2006, **281**, 32526–33.

52. S. Patel, R. George, F. Autore, F. Fraternali, J. E. Ladbury, and P. V Nikolova, *Nucleic Acids Res.*, 2008, **36**, 5139–51.

53. A. Friedler, L. O. Hansson, D. B. Veprintsev, S. M. V Freund, T. M. Rippin, P. V Nikolova, M. R. Proctor, S. Rüdiger, and A. R. Fersht, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 937–42.

54. N. Issaeva, A. Friedler, P. Bozko, K. G. Wiman, A. R. Fersht, and G. Selivanova, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 13303–7.

55. U. Moll and N. Slade, *Mol. Cancer Res.*, 2004, **2**, 371–386.

56. M. Levrero, V. De Laurenzi, A. Costanzo, J. Gong, J. Y. Wang, and G. Melino, *J. Cell Sci.*, 2000, **113**, 1661–70.

57. D. Bergamaschi, Y. Samuels, B. Jin, S. Duraisingham, T. Crook, and X. Lu, *Mol. Cell. Biol.*, 2004, **24**, 1341–1350.

58. P. Canning, F. von Delft, and A. N. Bullock, *J. Mol. Biol.*, 2012, **423**, 515–27.

59. R. A. Robinson, X. Lu, E. Y. Jones, and C. Siebold, *Structure*, 2008, **16**, 259–68.

60. A. Karnoub and R. Weinberg, *Nat. Rev. Mol. cell Biol.*, 2008, **9**, 517–531.

61. A. M. Rojas, G. Fuentes, A. Rausell, and A. Valencia, *J. Cell Biol.*, 2012, **196**, 189–201.

62. Y. Wang, N. Godin-Heymann, X. Dan Wang, D. Bergamaschi, S. Llanos, and X. Lu, *Cell Death Differ.*, 2013, **20**, 525–34.

63. Y. Wang, X. D. Wang, E. Lapi, A. Sullivan, W. Jia, Y.-W. He, I. Ratnayaka, S. Zhong, R. D. Goldin, C. G. Goemans, A. M. Tolkovsky, and X. Lu, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 13325–30.

64. N. Godin-Heymann, Y. Wang, E. Slee, and X. Lu, *PLoS One*, 2013, **8**, e82022.

65. X. D. Wang, E. Lapi, a Sullivan, I. Ratnayaka, R. Goldin, R. Hay, and X. Lu, *Cell Death Differ.*, 2011, **18**, 304–14.

66. Z. Wang and Y. Liu, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 312–317.

67. R. J. Youle and A. Strasser, *Nat. Rev. Mol. Cell Biol.*, 2008, **9**, 47–59.

68. N. Takahashi, S. Kobayashi, S. Kajino, K. Imai, K. Tomoda, S. Shimizu, and T. Okamoto, *Genes Cells*, 2005, **10**, 803–11.

69. C. Katz, H. Benyamini, S. Rotem, M. Lebendiker, T. Danieli, A. Iosub, H. Refaely, M. Dines, V. Bronner, T. Bravman, D. E. Shalev, S. Rüdiger, and A. Friedler, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 12277–12282.

70. T. D. Gilmore, *Oncogene*, 2006, **25**, 6680–4.

71. Y. Chen, W. Liu, L. Naumovski, and R. L. Neve, *J. Neurochem.*, 2003, **85**, 801–809.

72. H. Benyamini, H. Leonov, S. Rotem, C. Katz, I. T. Arkin, and A. Friedler, *Proteins*, 2009, **77**, 602–11.

73. S. Wen and S. F. Moss, *Cancer Lett.*, 2009, **282**, 1–8.

74. N. Pacchiani, S. Censini, L. Buti, and A. Covacci, *Cold Spring Harb. Perspect. Med.*, 2013, **3**.

75. L. L. Eftang, Y. Esbensen, T. M. Tannæs, I. R. K. Bukholm, and G. Bukholm, *BMC Microbiol.*, 2012, **12**, 9.

76. L. Buti, E. Spooner, A. G. Van der Veen, R. Rappuoli, A. Covacci, and H. L. Ploegh, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 9238–43.

77. N. R. Helps, H. M. Barker, S. J. Elledge, and P. T. Cohen, *FEBS Lett.*, 1995, **377**, 295–300.

78. S. Llanos, C. Royer, M. Lu, D. Bergamaschi, W. H. Lee, and X. Lu, *J. Biol. Chem.*, 2011, **286**, 43039–44.

79. M. P. Egloff, D. F. Johnson, G. Moorhead, P. T. Cohen, P. Cohen, and D. Barford, *EMBO J.*, 1997, **16**, 1876–87.

80. T. D. Skene-Arnold, H. A. Luu, R. G. Uhrig, V. De Wever, M. Nimick, J. Maynes, A. Fong, M. N. G. James, L. Trinkle-Mulcahy, G. B. Moorhead, and C. F. B. Holmes, *Biochem. J.*, 2013, **449**, 649–59.

81. A. Suzuki and S. Ohno, *J. Cell Sci.*, 2006, **119**, 979–87.

82. W. Cong, T. Hirose, Y. Harita, A. Yamashita, K. Mizuno, H. Hirano, and S. Ohno, *Curr. Biol.*, 2010, **20**, 1408–14.

83. R. Sottocornola, C. Royer, V. Vives, L. Tordella, S. Zhong, Y. Wang, I. Ratnayaka, M. Shipman, A. Cheung, C. Gaston-Massuet, P. Ferretti, Z. Molnár, and X. Lu, *Dev. Cell*, 2010, **19**, 126–37.

84. K. Janke, U. Brockmeier, K. Kuhlmann, M. Eisenacher, J. Nolde, H. E. Meyer, H. Mairbäurl, and E. Metzen, *J. Cell Sci.*, 2013, **126**, 2629–40.

85. H. Kim, G. Claps, a Möller, D. Bowtell, X. Lu, and Z. a Ronai, *Oncogene*, 2013, 1–7.

86. K. Siddle, *J. Mol. Endocrinol.*, 2011, **47**, R1–10.

87. F. Hakuno, S. Kurihara, R. T. Watson, J. E. Pessin, and S.-I. Takahashi, *J. Biol. Chem.*, 2007, **282**, 37747–58.

88. C. Liu, J. Luan, Y. Bai, Y. Li, L. Lu, Y. Liu, F. Hakuno, S.-I. Takahashi, C. Duan, and J. Zhou, *Gen. Comp. Endocrinol.*, 2014, **197**, 82–91.

89. H. Nakagawa, K. Koyama, and Y. Murata, *Cancer Res.*, 2000, **60**, 101–105.

90. A. S. Di, S. Strano, and G. Blandino, *Atlas Genet. Cytogenet. Oncol. Haematol.*, 2011, **14**, 1054–1058.

91. X. Espanel and M. Sudol, *J. Biol. Chem.*, 2001, **276**, 14514–23.

92. S. Gillotin and X. Lu, *FEBS Lett.*, 2011, **585**, 1778–82.

93. M. Irshad and I. Dhar, *Med. Princ. Pract.*, 2006, **15**, 405–16.

94. W.-T. Sun, P.-C. Hsieh, M.-L. Chiang, M.-C. Wang, and F.-F. Wang, *Biochem. Biophys. Res. Commun.*, 2008, **376**, 395–8.

95. C.-Y. Liu, X. Lv, T. Li, Y. Xu, X. Zhou, S. Zhao, Y. Xiong, Q.-Y. Lei, and K.-L. Guan, *J. Biol. Chem.*, 2011, **286**, 5558–66.

96. The PyMOL Molecular Graphics System, Version 1.3. Schrödinger, LLC.

97. F. E. Chen-Park, D.-B. Huang, B. Noro, D. Thanos, and G. Ghosh, *J. Biol. Chem.*, 2002, **277**, 24701–8.

98. T. Hayashi, M. Senda, H. Morohashi, H. Higashi, M. Horio, Y. Kashiba, L. Nagase, D. Sasaya, T. Shimizu, N. Venugopalan, H. Kumeta, N. N. Noda, F. Inagaki, T. Senda, and M. Hatakeyama, *Cell Host Microbe*, 2012, **12**, 20–33.

99. C. Chen, N. Gorlatova, Z. Kelman, and O. Herzberg, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 6456–61.

100. M. V Milburn, L. Tong, A. M. deVos, A. Brünger, Z. Yamaizumi, S. Nishimura, and S. H. Kim, *Science*, 1990, **247**, 939–45.

101. B. B. Touré, K. Miller-Moslin, N. Yusuff, L. Perez, M. Doré, C. Joud, W. Michael, L. DiPietro, S. van der Plas, M. McEwan, F. Lenoir, M. Hoe, R. Karki, C. Springer, J. Sullivan, K. Levine, C. Fiorilla, X. Xie, R. Kulathila, K. Herlihy, D. Porter, and M. Visser, *ACS Med. Chem. Lett.*, 2013, **4**, 186–190.

102. M. G. Hinds, M. Lackmann, G. L. Skea, P. J. Harrison, D. C. S. Huang, and C. L. Day, *EMBO J.*, 2003, **22**, 1497–507.

ASPP2 induces apoptosis and is downregulated in many types of cancer, making it a promising target for anti-cancer drugs.