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# New insights into the expanding complexity of the tumor suppressor ASPP2

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**Apoptosis Stimulating Protein of p53-2, ASPP2, aka 53BP2L, (encoded by *TP53BP2*) is a pro-apoptotic member of a family of p53 binding proteins. ASPP2 expression is frequently suppressed in human cancers and numerous studies have consistently demonstrated that ASPP2 inhibits cell growth as well as stimulates apoptosis—at least in part through a p53-mediated pathway. Two independent mouse models have shown that ASPP2 is a haplo-insufficient tumor suppressor and underscore the importance of the role of ASPP2 in human cancer. However, mounting evidence suggests that the mechanism(s) of action for ASPP2 are complex and likely extend beyond stimulation of apoptotic programs. Data highlighting this expanding spectrum of potential ASPP2-mediated pathways is summarized along with new results from recent in vivo models suggesting new avenues for investigation.**



## Introduction

ASPP2, aka 53BP2L, (encoded by *TP53BP2*) is a member of a family of proteins that shares as a common feature an Ankyrin repeat domain, an SH3 domain, and a Poly-Proline rich domain at the C-terminus (hence the ASPP family name emphasizes this feature).[1-3] In 1994 Iwabuchi and colleagues discovered the C-terminus of ASPP2 (originally called 53BP2) during a yeast two-hybrid screen using the wild-type p53 core domain as bait.[1] Shortly thereafter the C-terminal portion of 53BP2 was co-crystallized with the p53 core domain and resolved at 2.2 Å resolution by Gorina and Pavletich.[4] Most interestingly, the p53 "hotspot" mutations found in human cancers disrupt the 53BP2/p53 interaction[4-6]—suggesting that this interaction plays an important role in p53 biology. Armed with this structural knowledge, the great challenge over the last 15 years has been to understand the function(s) of ASPP2 (and family members).

In 1996, during a yeast two-hybrid screen using Bcl-2 as bait, Naumovski and Cleary found that 53BP2 was actually a partial clone of a transcript encoding a protein they named BBP (Bcl-2 Binding Protein), and that it inhibited cell growth when overexpressed.[7] Subsequent reports consistently demonstrated that Bbp/53BP2 inhibits cell growth: in 1997, Iwabuchi et al. found that overexpressed Bbp/53BP2 stimulates p53-mediated transcription and inhibits Ras/E1A-mediated transformation of rat embryonic fibroblasts (REFs);[8] and in 1999, Yang and colleagues found that Bbp/53BP2 overexpression induces apoptosis.[9] Nevertheless, the caveats of overexpression made it challenging to fully appreciate the significance of these observations. The in vitro binding promiscuity of the 53BP2 C-terminal domain has resulted in a number of reports describing its interaction with a variety of proteins[7,9-20] (**Table 1**). Although highly intriguing, the functional implications of many of these interactions have remained enigmatic. In 2000, Lopez et al. demonstrated that endogenous ASPP2 is damage-inducible, and that attenuation of endogenous ASPP2 expression promotes cell survival after damage—implying that

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**Table 1.** ASPP2 and/or 53BP2S potential functions and putative interacting partners

| Putative interactor | Putative functions or pathway interactions | Reference(s) |
|---|---|---|
| | *Modulates functions/pathways:* | |
| p53 (p73/p63) | Enhances p53 transcriptional activity/Promotes p53-mediated apoptosis | 2, 8, 17 |
| Bcl-2 (Bcl-$X_L$) | Impedes cell cycle progression/Induces mitochondrial-mediated apoptosis | 7, 54, 55 |
| IRS-1 | Modulates insulin signaling mediated by IRSs | 16 |
| dCsk | Drosophila ASPP interacts with dCsk to regulate dSrc kinase | 56 |
| APP-BP1 | Inhibits neddylation pathway via interaction with APP-BP1 | 14 |
| PP1 | Inhibits Protein Phosphatase 1 activity | 12 |
| | *Functions modulated by:* | |
| NFκB/p65 subunit | Apoptosis inhibited by NFκB pathway | 9, 55 |
| HCV core protein | Apoptosis inhibited by HCV core protein | 13 |
| Ddx42p | Apoptosis/cell growth suppression inhibited by DEAD box protein Ddx42p | 18 |
| DDA3 | Stimulation of p53-mediated BAX activation inhibited by DDA3 | 20 |
| | *Undefined functional association:* | |
| YAP | Phosphorylation by c-Yes inhibits interaction with YAP (a p73 co-activator) | 11 |
| APCL | Intracellular localization modulated by APCL | 10 |
| 14-3-3s | Associates with 14-3-3s during interphase | 15 |
| **Regulation of ASPP2 expression** | | |
| Epigenetic | Promoter methylation can silence transcription | 34, 35 |
| Transcriptional | Induced by activating E2Fs | 26, 42, 43 |
| Posttranscriptional | Splice isoform can truncate N-terminus | 3 |
| Posttranslational | Controlled by proteasomal degradation | 27 |



a biologic function of endogenous ASPP2 modulates physiologic damage response pathways.[21] Samuels-Lev and colleagues further opened the door by making the important finding that ASPP2 was a longer version of BBP, as well as a member of a new family of proteins that includes the pro-apoptotic ASPP1 and the anti-apoptotic iASPP (with all three family members encoded on different human chromosomes).[2,22-24] ASPP2 and BBP/53BP2 are splice isoforms,[3] with ASPP2 also referred to as 53BP2L (long), and BBP also referred to as 53BP2S (short). Consistent with the crystal structure[4] implicating functional interplay with the p53 pathway, Samuels-Lev et al. demonstrated that ASPP2 (and ASPP1) stimulated p53-mediated apoptosis (and hence, ASPP is also referred to as Apoptosis Stimulating Protein of p53).[2]

The p53 pathway is well known as a central player in the cellular stress response and tumor suppression.[25] Cytotoxic stress (such as DNA damage) or deregulated growth (such as activated oncogenes) signal to p53, whereupon it is activated to mediate different biologic pathways. Depending on the cell context and nature of stress, these pathways can range from cell cycle arrest and DNA repair, to induction of apoptosis or senescence. However the complex molecular mechanisms that regulate these different outcomes remain unclear and are the object of intensive investigation by a large number of laboratories. The finding that ASPP proteins modulate p53 (and p73/p63) apoptotic function,[2,17,22] places the ASPP family within an important molecular network that plays a critical role in tumorigenesis and response to therapy. The discovery that *ASPP2* is a direct E2F target gene further suggests it functions as a common link between the Rb/E2F and p53 pathways.[26] Control of ASPP2 protein levels by proteasomal degradation also modulates p53 function, adding yet another layer of complexity to the physiologic role for ASPP2 in the cellular damage response.[27]

## ASPP2 is a Tumor Suppressor

Clinical studies have revealed low ASPP2 expression in human tumors and correlation with poor clinical outcome.[2,28-33] Although tumor derived ASPP2 mutations have not been published, epigenetic silencing of ASPP2 by promoter methylation may be an important mechanism for suppressing ASPP2 expression.[34,35] Interestingly, single nucleotide polymorphisms (SNPs) at the *TP53BP2* locus are associated with gastric cancer susceptibility.[36] Such clinical observations, along with the in vitro findings demonstrating ASPP2 and/or Bbp/53BP2S inhibits cell growth, promotes apoptosis and inhibits Ras/E1A mediated transformation[2,8,9,21,22,37] provided the rationale for the hypothesis that ASPP2 could function as a tumor suppressor.

Two mouse models targeting *ASPP2* using homologous recombination have now tested this hypothesis, and both demonstrate that ASPP2 has in vivo tumor suppressor function.[38,39] Vives and colleagues found that although *ASPP2*$^{-/-}$ mice die before weaning, *ASPP2*$^{+/-}$ mice have an increased incidence of spontaneous and γ-irradiation-induced tumors without loss of heterozygosity at the remaining *ASPP2* allele—consistent with the notion that ASPP2 is a haplo-insufficient tumor suppressor.[38,40] In this *ASPP2* mouse model, genetic evidence further suggests developmental cooperation with the p53 pathway since postnatal lethality is enhanced in a

*p53*$^{+/-}$ background and homozygous deletion of both *p53* and *ASPP2* is synthetic lethal.[38] Genetic cooperation with p53 is also seen with tumor suppression since *ASPP2*$^{+/-}$;*p53*$^{+/-}$ mice develop tumors at an overall higher frequency compared to *ASPP2*$^{+/+}$;*p53*$^{+/-}$ mice.[38] This appears to be tumor-type-specific as accelerated sarcoma development is not seen in these compound heterozygotes.[38] Moreover, accelerated lymphoma development is apparent at 42 weeks, but not at 72 weeks.[38] Together, this perhaps hints that other p53-independent pathways might also genetically cooperate with *ASPP2*.

Kampa and colleagues also found evidence that ASPP2 is an in vivo tumor suppressor by targeting *ASPP2* and observing that the development of spontaneous tumors, as well as γ-irradiation-induced high-grade thymic lymphomas, is accelerated in *ASPP2*$^{+/-}$ mice.[39] Given the modest affects from the loss of a single *ASPP2* allele and the long interval required to develop tumors in these haplo-insufficient mice, it is gratifying that both ASPP2 mouse models have similar spontaneous and γ-irradiation-induced tumor-free survival curves.[38,39] It is notable, however, that there are differences between these models. Vives et al. find their *ASPP2*$^{-/-}$ mice are born at a lower than expected frequency and die prior to weaning likely due to a combination of heart defects and hydrocephalus.[38] In contrast, Kampa and colleagues do not identify *ASPP2*$^{-/-}$ embryos even as early as embryonic day 6.5, regardless of background *p53* genotype, nor in an inbred Balb/c backround.[39] Moreover, Kampa et al. do not observe accelerated tumorigenesis in *ASPP2*$^{+/-}$;*p53*$^{+/-}$ mice (compared to *ASPP2*$^{+/+}$;*p53*$^{+/-}$ mice).[39] It is possible that subtle strain specific modifiers could account for these differences, or that the negative *ASPP2*$^{+/-}$;*p53*$^{+/-}$ experiment was underpowered and unable to detect only modest differences in tumor latency.[39] Nevertheless, while these differences between mouse models remain to be elucidated, they may ultimately provide an opportunity to shed new light on ASPP2 biology. Indeed, the literature is replete with examples of different mouse models, targeting the same allele but resulting in variable phenotypes, which have ultimately proven instructive. Vives et al. targeted *ASPP2* exon 3,[38] while Kampa et al. targeted *ASPP2* exons 10-17.[39] Even though reduction in the levels of ASPP2 protein is found in both mouse models, it should be pointed out that *ASPP2* is an 18 exon gene spanning approximately 50 kB (including an approximately 25 kB first intron). Hence, it is quite possible that the different targeting strategies disrupt different—and yet to be defined—regulatory (or other) elements within the complex *ASPP2* locus.

It is instructive to also consider that the homologous family member ASPP1 has indistinguishable in vitro pro-apoptotic functions from ASPP2,[2,17]—yet does not compensate for the developmental defects resulting from the targeting of both ASPP2 alleles, or the accelerated tumor formation caused by targeting one ASPP2 allele.[38,39] Conversely, ASPP2 does not compensate for the developmental defects in lymphatic vessel formation of *ASPP1*$^{-/-}$ mice; nor is this defective lymphatic development altered in a *p53*$^{+/-}$ or *p53*$^{-/-}$ background.[41] Moreover, even though *ASPP1*$^{-/-}$ mice reach adulthood and reproduce, they have not been reported as tumor prone.[41] This underscores the limitations of in vitro studies and the importance of in vivo models to uncover new avenues for investigation into the complex function(s) of the ASPP family.

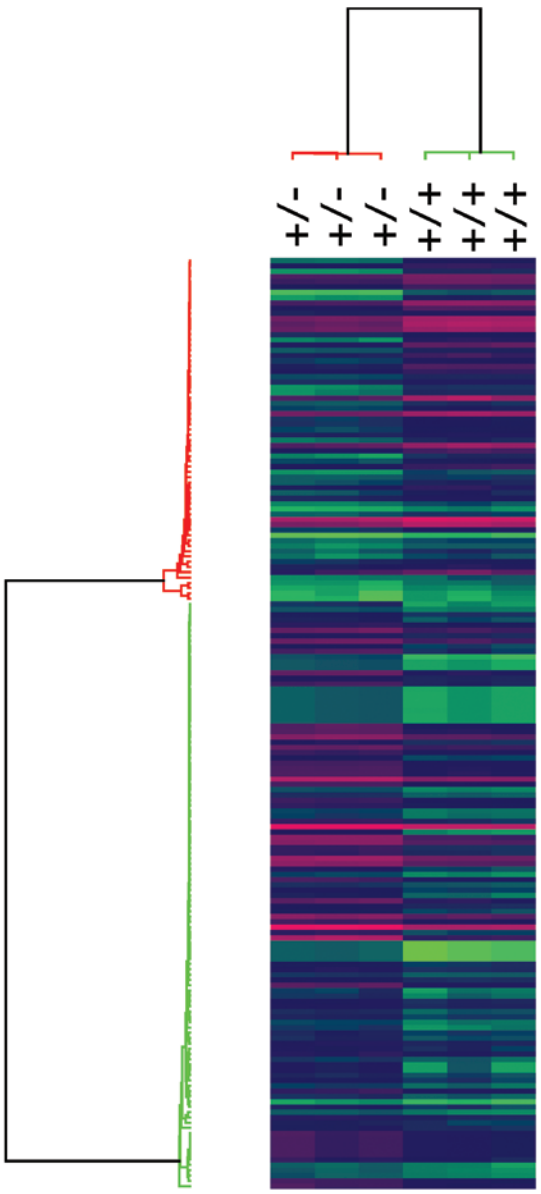


## ASPP2 May Have Functions Beyond Enhancing p53-Mediated Apoptosis

Kampa et al. additionally found that *ASPP2*$^{+/-}$ primary thymocytes (derived from the exon 10-17 targeted mouse) have an attenuated apoptotic response after ex vivo γ-irradiation.[39] This suggests a potential mechanism for accelerated thymic lymphomagenesis in γ-irradiated *ASPP2*$^{+/-}$ mice since reduced apoptosis may result in the persistence of thymocytes harboring tumorigenic mutations. However, perhaps the most intriguing findings from this mouse are the suggestions of ASPP2 function beyond p53-mediated apoptosis. Kampa et al. find that γ-irradiated *ASPP2*$^{+/-}$ primary mouse embryonic fibroblasts (MEFs) (which normally undergo a $G_0/G_1$ cell cycle arrest versus apoptosis) have a defective $G_0/G_1$ checkpoint—tantalizingly suggesting that ASPP2 function is far more complex than simply enhancing p53 pro-apoptotic transcriptional programs.[39] Although the molecular mechanisms underlying these attenuated cellular damage-response thresholds remain to be clarified, it seems that cell type and context are important determinants of when/if ASPP2 engages different biologic programs that ultimately inhibit cell growth and promote tumor suppression. There is ample hypothesis-generating in vitro evidence in the literature suggesting that ASPP2 (and/or isoforms) also modulates expression of cell cycle-regulating genes, perturbs cell cycle progression, binds and modulates other proteins, or is regulated by pathways involved in diverse cellular functions[7,9-20,26,42,43] (**Table 1**).

Recent structural insights on ASPP2 are in-line with the concept that modulation of p53-dependent apoptosis is complex, and only a part of the story. The C-terminus, containing the signature proline-rich, ankryrin-repeat and SH3 domain, is highly conserved amongst all of the ASPP family members.[23] Based on subtle structural differences, these C-terminal domains from the ASPP family members appear to variably bind p53 family members (and other proteins). These findings may eventually prove to have important functional, as well as therapeutic, implications.[19,44-51] In contrast to the conserved C-terminal domain, the ASPP2 N-terminus is only conserved with ASPP1 (not iASPP), which suggests that this domain may harbor unique function(s).[23,47,52] The intracellular localization of ASPP2 adds another dimension to consider when contemplating potential ASPP2 function(s). Full-length ASPP2 localizes predominantly in the cytoplasm, while the C-terminus localizes predominantly to the nucleus.[9,53] It has been reported that the 53BP2S isoform of ASPP2 localizes to mitochondria and modulates the mitochondrial death pathway[54,55]—an intriguing observation given the potential interaction with Bcl-2 family proteins.[7,19] The ASPP2 N-terminus is subject to alternative splicing—raising the possibility that the N-terminus contains important domains that need to be regulated in order to restrict ASPP2 function to the proper cellular or developmental context.[3] Interestingly, the solution structure of

A




B

| Gene symbol | γ–irradiation fold-change (+/- vs +/+) | |
|---|---|---|
| 10379987* | 2.317 | up |
| CTSE | 2.314 | up |
| 10394931* | 2.303 | up |
| PBP2 | 2.129 | up |
| EG622147 | 2.111 | up |
| 10378781* | 2.11 | up |
| 10378781* | 2.11 | up |
| 10362111* | 2.109 | up |
| AMACR | 2.041 | up |
| JAM2 | 1.925 | up |
| 10347218* | 1.869 | up |
| ADAMTS5 | 1.821 | up |
| TMEM16A | 1.816 | up |
| CXCR6 | 1.796 | up |
| TBX20 | 1.79 | up |
| GABRB1 | 1.777 | up |
| PTPRZ1 | 1.752 | up |
| PCDH9 | 1.751 | up |
| IQGAP2 | 1.75 | up |
| Sult4a1 | 1.745 | up |
| FIBIN | 1.731 | up |
| VCAN | 1.723 | up |
| MPP7 | 1.724 | up |
| HMX1 | 1.724 | down |
| GAP43 | 1.721 | up |
| PCDH9 | 1.715 | up |
| RNU2-1 | 1.714 | up |
| DACT1 | 1.707 | up |
| OLFR111 | 1.675 | up |
| F2RL2 | 1.675 | down |
| MMP16 | 1.671 | up |
| TGFBI | 1.656 | up |
| CLCA1 | 1.653 | up |
| SORBS2 | 1.647 | up |
| MS4A4A | 1.646 | up |
| ECE1 | 1.645 | up |
| ATF7IP | 1.635 | up |
| CD34 | 1.632 | up |
| PLAGL1 | 1.626 | up |
| 10429968* | 1.623 | up |
| 10564015* | 1.623 | down |
| 10549530* | 1.616 | up |
| PIGN | 1.613 | up |
| LMO7 | 1.611 | up |
| ELOVL7 | 1.605 | up |
| C6ORF118 | 1.599 | down |
| IFITM1 | 1.596 | down |
| PMP2 | 1.585 | down |
| TMC7 | 1.581 | up |
| KLHDC8A | 1.579 | up |
| HCN1 | 1.575 | up |
| JAG1 | 1.57 | up |
| PDLIM2 | 1.567 | up |
| SCUBE3 | 1.564 | up |
| RNF122 | 1.563 | up |
| FLRT3 | 1.552 | up |
| UNC13C | 1.548 | down |
| LCE1I | 1.536 | up |
| USP18 | 1.533 | down |
| 10395908* | 1.532 | up |
| V1RD21 | 1.521 | down |
| PKP1 | 1.516 | up |
| RRAS2 | 1.516 | up |
| RGS2 | 1.516 | up |
| 10550760* | 1.514 | down |
| ATP2A3 | 1.513 | down |
| ARC | 1.513 | down |
| CLCA2 | 1.511 | up |

**Figure 1.** Microarray expression profiling of γ-irradiated *ASPP2*$^{+/-}$ and *ASPP2*$^{+/+}$ MEFs demonstrates significant gene expression differences. (A) Heatmap of global expression differences between triplicate pairs of early passage *ASPP2*$^{+/-}$ and *ASPP2*$^{+/+}$ MEFs that were γ-irradiated with 5 Gy and harvested 12 hours later. *ASPP2*$^{+/-}$ MEFs are in columns 1–3 and *ASPP2*$^{+/+}$ MEFs are in columns 4–6. (B) Top scoring genes that were differentially expressed between *ASPP2*$^{+/-}$ and *ASPP2*$^{+/+}$ MEFs after γ-irradiation. Statistically significant fold-change differences in gene expression between γ-irradiated *ASPP2*$^{+/-}$ MEFs (relative to γ-irradiated *ASPP2*$^{+/+}$ MEFs) are shown, and do not include genes that were differentially expressed between the genotypes at baseline. *denotes probe set ID/transcript cluster of non-annotated genes found on Affymetrix Mouse Gene ST 1.0 array.

the ASPP2 N-terminus reveals a β-Grasp ubiquitin-like fold, as well as a potential Ras-binding (RB)/Ras-association (RA) domain.[52] Taken together, all of these observations suggest that ASPP2 has important functions within the cytoplasmic compartment—and it is tempting to speculate that these may be mediated via the N-terminus.

Because ASPP2 is a bonafide tumor suppressor that may do more than stimulate apoptosis, we wished to explore how attenuated ASPP2 expression affected global molecular networks. We therefore performed global gene expression profiling on early passage *ASPP2*$^{+/+}$ and *ASPP2*$^{+/-}$ MEFs. As shown in **Figure 1**, we found significant gene expression differences between γ-irradiated *ASPP2*$^{+/+}$ and *ASPP2*$^{+/-}$ MEFs. Although the significance of these findings requires further functional validation, this example of unique genomic expression profiles using genetically defined systems may provide a glimpse into potential new pathways and mechanisms of ASPP2 function(s). Global posttranslational interrogation (such as the phosphoproteome) of these genetically defined systems may also open new avenues for investigation. Indeed, the drosophila homologue dASPP regulates C-terminal Src Kinase activity,[56] and the 53BP2S isoform of ASPP2 modulates insulin signals mediated by insulin receptor substrates[16]—suggesting that ASPP2 affects cellular functions by modulating complex signal transduction networks independent of any ability to enhance p53-mediated transcription.

## Summary

The function of ASPP2 as a haplo-insufficient tumor suppressor has been shown in two different mouse models using two different targeting strategies.[38,39] These results rigorously confirm the mounting evidence for the role of ASPP2 as a tumor suppressor in human cancer.[2,28-34,36] Yet, as frequently seen in biology, more unanswered questions remain and more importantly, many new and exciting questions have now been posed. Our understanding of the increasing complexity of ASPP2 (and family members) has only just begun to scratch the surface. No doubt, it will be fascinating to see how these yet to be discovered ASPP2 functions will shed new light on cancer biology, and ultimately reveal new targets for therapy in the oncology clinic.

## Methods

Briefly, total RNA was isolated from MEFs using the RNeasy® Mini Kit with a column DNAase digestion step (Qiagen). RNA quality was checked by a Lab-on-a-Chip-System Bioanalyzer 2100 (Agilent). Biotin-labeled, fragmented cDNA was generated per standard protocol (Genechip® WT Sense Target Labeling Assay) and hybridized to Affymetrix Genechip® Mouse Gene ST 1.0 arrays that were automatically washed and stained with streptavidin-phycoerythrin using a fluidics station. The probe arrays were scanned at a 1.4-μm resolution using an Affymetrix Genechip® 3000 System scanner. Raw intensities from the CEL files were analyzed using Affymetrix Power Tool (APT, version 1.8.5) to generate robust multi-array average intensity in log2 scale for each probe set. The perfect match intensities for each probe set were corrected for background and quantile-normalized using a robust fit of linear models. Differential expression was determined using ArrayAssist software 5.5 (Agilent). Microarray analysis was performed at the Microarray Facility Tübingen, Universität Tübingen, Tübingen Germany.



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