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# ΔN-ASPP2, a novel isoform of the ASPP2 tumor suppressor, promotes cellular survival

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## Abstract

ASPP2 is a tumor suppressor that works, at least in part, through enhancing p53-dependent apoptosis. We now describe a new ASPP2 isoform, ΔN-ASPP2, generated from an internal transcription start site that encodes an N-terminally truncated protein missing a predicted 254 amino acids. ΔN-ASPP2 suppresses p53 target gene transactivation, promoter occupancy, and endogenous p53 target gene expression in response to DNA damage. Moreover, ΔN-ASPP2 promotes progression through the cell cycle, as well as resistance to genotoxic stress-induced growth inhibition and apoptosis. Additionally, we found that ΔN-ASPP2 expression is increased in human breast tumors as compared to adjacent normal breast tissue; in contrast, ASPP2 is suppressed in the majority of these breast tumors. Together, our results provide insight into how this new ASPP2 isoform may play a role in regulating the ASPP2-p53 axis.

### Keywords

ASPP2; Tumor suppressor; p53

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## 1. Introduction

The p53 pathway is a central player in regulation of both cellular stress response and tumor suppression [1]. Not surprisingly, p53 is one of the most highly mutated genes in human cancer. However, in some cancers the frequency of p53 mutations is relatively low (~30% [2]). Thus, functional inactivation of the p53 pathway must occur by other mechanisms besides p53 mutation.

ASPP2 is a member of a family of p53-binding proteins that share homology in their C-terminus. The ankyin-repeat and SH3 domains of ASPP2 bind the p53 core domain [3] and modulate p53 function [3–5]. The full-length 1134 a.a. ASPP2, and to a lesser extent the 1005 a.a. splice variant BBP [6,7], stimulate p53-mediated transcription, inhibit cell growth, and promote apoptosis [3,4]. ASPP2 selectively stimulates p53 transactivation of target genes [4] but also mediates p53-independent functions [3,8]. Targeting *ASPP2* in mouse models demonstrates tumor suppressor function [9,10] and clinical studies demonstrate reduced *ASPP2* expression in human tumors [4,11–18]. Not surprisingly giving its complex functions, ASPP2 expression is also complex [3,19] with the 18 exon *ASPP2* locus spanning over 50 kilobases.

The ASPP2 N-terminus contains important functional domains [20]. The natural occurring N-terminally truncated ASPP2 splice isoform BBP has attenuated function compared to full-length ASPP2 including decreased apoptosis and growth-inhibitory functions [4]. However, little is known about other ASPP2 N-terminal truncated isoforms or their functions.

## 2. Experimental methods

### 2.1. Cell lines

All cells were maintained as previously described [21]. Tetracycline-regulatable FLAG-ASPP2 and FLAG-ΔN-ASPP2 cell lines were generated by transfection followed by Zeocin™selection at 100 μg/mL. HCC202 and DU4475 cell lysates were a gift from Dr. Trevor Levin and Dr. Joe Gray (Oregon Health & Science University). HCT116 isogenic cell lines were a gift from Dr. Bert Vogelstein (Johns Hopkins University).

### 2.2. ΔN-ASPP2 cloning

Total RNA was isolated from cells using TRIzol® according to manufacturer's instructions. Samples were DNase treated for 30 min at 37 °C. cDNA was generated using M-MLV Reverse Transcriptase using an oligo-dT primer. Undiluted cDNA was used with Platinum Taq polymerase with the annealing temperature optimized to 72 °C for 35 cycles. Purified products were ligated into a TOPO TA Cloning Kit® and sequenced with cloning primers (Fig. S1).

### 2.3. 5′RACE

Total RNA was collected from fresh mouse brain tissue and used in the GeneRacer™ 5′ RACE System along with *ASPP2*-specific reverse primers (Fig. S2).

### 2.4. qRT-PCR, and primer/probe sequences

Total RNA was isolated and cDNA was synthesized using a High Capacity cDNA Reverse Transcriptase Kit. Samples were run with the specified primers (Fig. S2) using TaqMan® reagents and StepOne™ Real-Time PCR system. Human GAPDH was used for normalization. Patient matched normal and breast cancer samples were collected from women undergoing mastectomies, after written and informed consent was obtained as approved by the Shandong Tumor Hospital Institutional Review Board and Ethics Committee, P.R.C. De-identified cDNA was prepared by authors DC and ZY.

### 2.5. Immunoblotting and antibodies

Immunoblot analysis was performed as described previously [22,23]. The N-terminal ASPP2 antibody was from Abcam. The C-terminal ASPP2 antibody and FLAG antibody was from Sigma-Aldrich.

### 2.6. Luciferase assay

Luciferase assay was performed as described previously [22].

### 2.7. ChIP

FLAG-ASPP2-tr-U2OS or FLAG-ΔN-ASPP2-tr-U2OS cells were induced with doxycycline for 18 h and then exposed to 20 μM cisplatin or 0.9% NaCl for 4 h. ChIP was performed as described previously with 2 μg α-p53 (DO-1) or IgG [22].

### 2.8. Annexin V staining

Annexin V staining was performed as described previously [10].

### 2.9. Live cell imaging

Cells were plated at a density of 1500 cells/well in a 96-well plate. Twenty-four hours later the percent cell confluence over time was determined every 2 h for 48 h using an Incucyte ZOOM™ automated microscope.

### 2.10. MTS assay

Cells were plated at a density of 1500 cells/well in a 96-well plate and 24 h later an MTS proliferation assay was performed according to manufacturer's instructions.

### 2.11. RNAseq data

RNA collected from BRCA cell lines were converted into cDNA library fragments. Sequencing adaptors were added to each cDNA fragment and paired end sequencing was done using Illumina GAII. The reads were then aligned to reference genome build hg19 using Tophat [24,25], a splice junction aligner. Integrative Genomics Viewer [26] was used to view aligned reads.

## 3. Results

### 3.1. ΔN-ASPP2 is a novel N-terminal truncated isoform of ASPP2

To find unknown *ASPP2* gene products, we utilized 5′-RACE to detect mature capped *ASPP2* transcripts in mouse, human, and rat cDNA (Fig. 1A; Fig. S3). In addition to the known full-length *ASPP2* transcript [4], we identified and sequence-verified a novel *ASPP2* mRNA generated from an internal TSS within intron 6 (Fig. 1A). ΔN-ASPP2 is not a splice isoform of the full-length *ASPP2* transcript, since exon 1-initiatied RT-PCR only detects *ASPP2* and *BBP* mRNA in human and mouse (Fig. S4). We named the new isoform ΔN-ASPP2 since the predicted ATG in exon 8 would generate an N-terminal truncated 880 a.a. protein (Fig. 1B).

Sequencing of the 5′-RACE products identified a unique 5′-untranslated region in the mature *ΔN-ASPP2* mRNA. The genomic sequence was further analyzed using the promoter prediction software Promoter 2.0 [27] and revealed a high scoring TSS (score 1.071) that is within 380 base pairs of our experimentally determined ΔN-ASPP2 TSS (Fig. S5).

### 3.2. ΔN-ASPP2 is expressed in cells

To confirm that the newly identified TSS generated an intact ΔN-ASPP2 mRNA, we cloned and sequenced the entire ΔN-ASPP2 cDNA using a 5′-UTR-specific forward primer and 3′UTR-specific reverse primer (Fig. S6). We did not detect mutations in the shared open reading frame with ASPP2. Using a C-terminal specific ASPP2 antibody on DU4475 and HCC202 cell lysates, we detected an endogenous ASPP2-immunoreactive protein ~125 kD in size that migrated faster than the ~165 kD ASPP2 protein (Fig. 1C, lanes 3 and 4). It is known that ASPP2 migrates anomalously slow (~165 kD as compared to predicted ~135 kD) due to its polyproline rich domain [4]. This domain is conserved in ΔN-ASPP2 (Fig. 1B), which would account for ΔN-ASPP2 migrating more slowly than predicted (~125 kD as compared to predicted ~90 kD). Epitope mapping was used to confirm the ~125 kD endogenous band is ΔN-ASPP2. The band was only recognized by a C-terminal ASPP2 antibody (a.a. 691-1128), but not an N-terminal ASPP2 antibody (a.a. 50–150; (Fig. 1C, lanes 1, 2 vs 3, 4). These results were repeated with a ΔN-ASPP2 expression vector (Fig. 1D).

Finally, to provide additional support that ΔN-ASPP2 is expressed *in vivo*, we analyzed previously described $ASPP2^{exon10–17/+}$ MEFs [10] and found reduced *ΔN-ASPP2* expression as compared to $ASPP2^{+/+}$ MEFs (Fig. 1E). Additionally, using an unbiased RNA-seq database created from breast cancer cell lines, we detected the presence of the 5′UTR sequence that is unique to ΔN-ASPP2 in the human tumor cell lines HCC202, DU4475, SUM159PT, and 21MT1 (Fig. S7). Together, these results confirm the existence of a new N-terminal truncated ASPP2 isoform.

### 3.3. ΔN-ASPP2 inhibits p53 transcriptional activation

Since the N-terminal truncated ASPP2 splice isoform BBP has a reduced ability to enhance p53-mediated apoptosis, and the partial transcript 53BP2 is dominant-negative against p53 and ASPP2 [4,6], we reasoned that ΔN-ASPP2 could oppose p53 function. To explore this, we quantified p53 transactivation activity in the p53 null cell line H1299. We determined that ΔN-ASPP2 inhibited exogenous p53 transactivation of the *p21*-luciferase reporter in a dose dependent manner (Fig. 2A). We next tested if ΔN-ASPP2 could inhibit endogenous p53 transactivation function using the isogenic HCT116p53$^{+/+}$ and HCT116p53$^{-/-}$cell lines [28]. When ΔN-ASPP2 was expressed in HCT116p53$^{+/+}$ cells, there was a 50% reduction in p53-stimulation of the *p21*-luciferase reporter as compared to p53-stimulation alone (Fig. 2B).

To provide mechanistic insight into how ΔN-ASPP2 attenuates p53 transactivation, we performed quantitative chromatin immunoprecipitation of endogenous p53 in U2OS cells with tetracycline-inducible FLAG-ASPP2 or FLAG-ΔN-ASPP2 (Fig. 2C, boxed inset). As expected [4], cisplatin and ASPP2 expression increased endogenous p53 protein binding at the *Bax* promoter >4-fold as compared to control (Fig. 2C top panel). In contrast, cisplatin and ΔN-ASPP2 expression did not increase p53 at the *Bax* promoter (Fig. 2C, top panel). ΔN-ASPP2 expression also reduced p53 occupancy at the *p21* promoter after cisplatin treatment as compared to cells expressing ASPP2 (Fig. 2C bottom panel). Consistent with these results, we found that ΔN-ASPP2 expression inhibited doxorubicin-induced stimulation of endogenous *p21* mRNA (Fig. 2D). Together, these results demonstrate that ΔN-ASPP2 and ASPP2 have opposing effects on p53 target gene activation and promoter occupancy.

### 3.4. ΔN-ASPP2 enhances cell proliferation and survival

To explore the biological consequences of ΔN-ASPP2 inhibition of p53 transactivation, we quantified proliferation in ΔN-ASPP2 and ASPP2-inducible U2OS cell lines (Fig. 3A). After induction of ΔN-ASPP2, we found an increased number of cells as compared to non-induced cells (Fig. 3A, 29.0% verses 16.3% confluence). As a control and as predicted, ASPP2 expression inhibited cell proliferation as compared to non-induced cells (Fig. 3A, 33.6% confluence verses 20.1%). When ΔN-ASPP2 was expressed in cells and then exposed to cisplatin, we found they were more resistant to cisplatin growth inhibition when compared to un-induced cells (Fig. 3B, top panel). Conversely, we noted a decrease in proliferation in cells expressing ASPP2 as compared to un-induced cells (Fig. 3B, bottom panel). Additionally, when cells expressing ΔN-ASPP2 were exposed to cisplatin for 24 h, cell viability was not decreased at 5 μM, and only a modest decrease was seen at 25 μM (Fig. 3C, left panel). In contrast and as expected, expression of full-length ASPP2 showed inhibition of cell viability alone and in combination with cisplatin (Fig. 3C, right panel). Together these data demonstrate a novel biologic function of ΔN-ASPP2 to promote proliferation and viability, which is in contrast to ASPP2 function [4,29].

### 3.5. ΔN-ASPP2 inhibits damage-induced apoptosis

Since the ASPP2 N-terminus is important for UV-induced apoptosis [4,10], we reasoned that ΔN-ASPP2 would inhibit UV-induced apoptosis. ΔN-ASPP2 significantly inhibited UV-induced apoptosis in U2OS cells exposed to 40 J/m$^2$ UVC. (Fig. 3D). This is in contrast to prior findings that ASPP2 promotes UV-induced apoptosis [4,29]. Together, these results demonstrate that ΔN-ASPP2 inhibits damage-induced apoptosis.

### 3.6. ΔN-ASPP2 is overexpressed in breast tumors

Since ΔN-ASPP2 promotes cell proliferation and survival (Fig. 3), we determined to what extent ΔN-ASPP2 was over- expressed in human cancer. *ΔN-ASPP2* expression was measured in matched breast tumor and adjacent normal breast tissue using *ΔN-ASPP2*-specific forward primers (Fig. 4A). Interestingly, *ΔN-ASPP2* expression was elevated compared to adjacent normal tissue. Conversely, *ASPP2* mRNA expression was suppressed in many of these breast cancer specimens compared to matched normal tissue (Fig. 4B), which is consistent with previous reports [4,14,16,30]. Relative *ASPP2* and *ΔN-ASPP2* expression across normal tissues did not exhibit wide variation to account for differences across tumors {*ΔN-ASPP2/GAPDH* mean = 0.034 (0.019–0.054), std. dev = 0.011; *ASPP2/GAPDH* mean = 0.812 (0.61–0.9), std. dev = 0.069}. Our findings that *ΔN-ASPP2* is overexpressed in human breast cancers suggest that it may play an important role in human tumorigenesis.

## 4. Discussion

Despite mouse models demonstrating that ASPP2 can function as a tumor suppressor [9,10], precisely how it does so and how it is regulated remain unclear. Our discovery of ΔN-ASPP2 provides significant insight into understanding the complex regulation and function of ASPP2. We have demonstrated that ΔN-ASPP2 is generated from an alternative TSS in the *ASPP2* locus (Fig. 1A) and that it is not a splice isoform (Fig. S4). Moreover, *ΔN-ASPP2* mRNA and protein expression can be detected in both human and mouse tissues (Fig. 1C, E and Fig. S6).

Our data suggest that ΔN-ASPP2 antagonizes the growth-inhibitory functions of ASPP2 and promotes survival (Fig. 3), which is consistent with prior reports [4]. ΔN-ASPP2 might contribute to differences between *ASPP2* targeted mouse models [9,10]. Our data that ΔN-ASPP2 is generated from an internal TSS (Fig. 1A) suggest that the $ASPP2^{+/\Delta exon10\text{–}17}$ targeting strategy disrupts the coding sequence for both ASPP2 and ΔN-ASPP2. The $ASPP2^{+/\Delta exon3}$ targeting strategy [10] would be predicted to not disrupt ΔN-ASPP2. Indeed, $ASPP2^{\Delta exon3/\Delta exon3}$ MEFs continue to express the BBP splice isoform [31]. Our findings emphasize the need for a clearer understanding of ASPP2 regulation and expression.

$ASPP2^{+/\Delta exon3}$;$p53^{+/-}$ mice cooperate with p53 by accelerating tumor formation [9]. In contrast, $ASPP2^{+/\Delta exon10\text{–}17}$;$p53^{+/-}$ mice accelerate tumor formation independent of p53 [10]. If $ASPP2^{+/\Delta exon3}$ mice leave ΔN-ASPP2 intact, its dominant-negative activity could further inhibit p53 to accelerate tumors. However targeting both ASPP2 and ΔN-ASPP2 in $ASPP2^{+/\Delta exon10\text{–}17}$ mice would attenuate ΔN-ASPP2 dominant-negative function, and thus mask genetic cooperation between ASPP2 and p53.

Our data that ΔN-ASPP2 can promote proliferation (Fig. 3A), inhibit the growth suppressive effects of cisplatin (Fig. 3B and C) and inhibit UV-induced apoptosis (Fig. 3D), are in direct contrast to ASPP2 functions [4,29]. ASPP2 promotes apoptosis in part through enhancing p53 transactivation—making it tempting to speculate that the pro-survival functions of ΔN-ASPP2 might be in part due to inhibiting p53 transcription. Indeed, we found that ΔN-ASPP2 inhibits endogenous p53 transactivation of a *p21*-luciferase reporter as well as inhibits damage-induced activation of endogenous *p21* mRNA (Fig. 2). Importantly, we confirmed by ChIP that ΔN-ASPP2 could directly inhibit endogenous p53 occupancy on the endogenous *p21* and *Bax* promoters in response to cell damage (Fig. 2C). It remains to be determined how ΔN-ASPP2 modulates p53 occupancy on target gene promoters. ASPP2 can directly bind p53 at the C-terminal domain preserved in ΔN-ASPP2 [4,32,33]. Thus, we could hypothesize that ΔN-ASPP2 also binds p53 to prevent it from occupying p53 target gene promoters or to compete with ASPP2 for p53 binding [4]. Thus ΔN-ASPP2 may also directly bind ASPP2 to inhibit its function as would be predicted by structural studies [34,35]. Intriguingly, we observed that expression of ΔN-ASPP2 appears to suppress expression of ASPP2 (Fig. 1C), supporting a role for ΔN-ASPP2 in ASPP2 inhibition. Since ΔN-ASPP2 would theoretically not interact with ASPP2 N-terminal binding partners [8,31,36–38] this may also play a role in its function. Our findings open the door for further study of these complex ΔN-ASPP2/ ASPP2 pathways.

Prior studies show that *ASPP2* expression is suppressed in breast cancer [4,14,16]. While informative, these studies did not distinguish between full-length *ASPP2* and *BBP* or *ΔN-ASPP2* transcripts. Our analysis of a series of breast cancer cases confirms decreased *ASPP2* tumor expression using *ASPP2* specific qRT-PCR (Fig. 4B) and we found that *ΔN-ASPP2* was overexpressed in these same breast cancer samples (Fig. 4A). These findings suggest that aberrant ΔN-ASPP2 expression might be important clinically.

Our discovery of ΔN-ASPP2 is significant because it sheds new light on prior ASPP2 studies relative to known p53 functions. However, mounting evidence demonstrates important p53-independent ASPP2 functions beyond cell survival and apoptosis [9,38]; [39–43] and it is likely that ΔN-ASPP2 will also play a role. Although the precise mechanisms remain to be elucidated, our findings that ΔN-ASPP2 is overexpressed in human cancers, promotes resistance to cell damage and enhances cell survival, makes it a potential target to be exploited for cancer therapy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **ASPP2** | Apoptosis-stimulating protein of p53 2 |
| **TSS** | transcription start site |
| **MEF** | mouse embryonic fibroblast |

**a.a** amino acid

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.12.027.

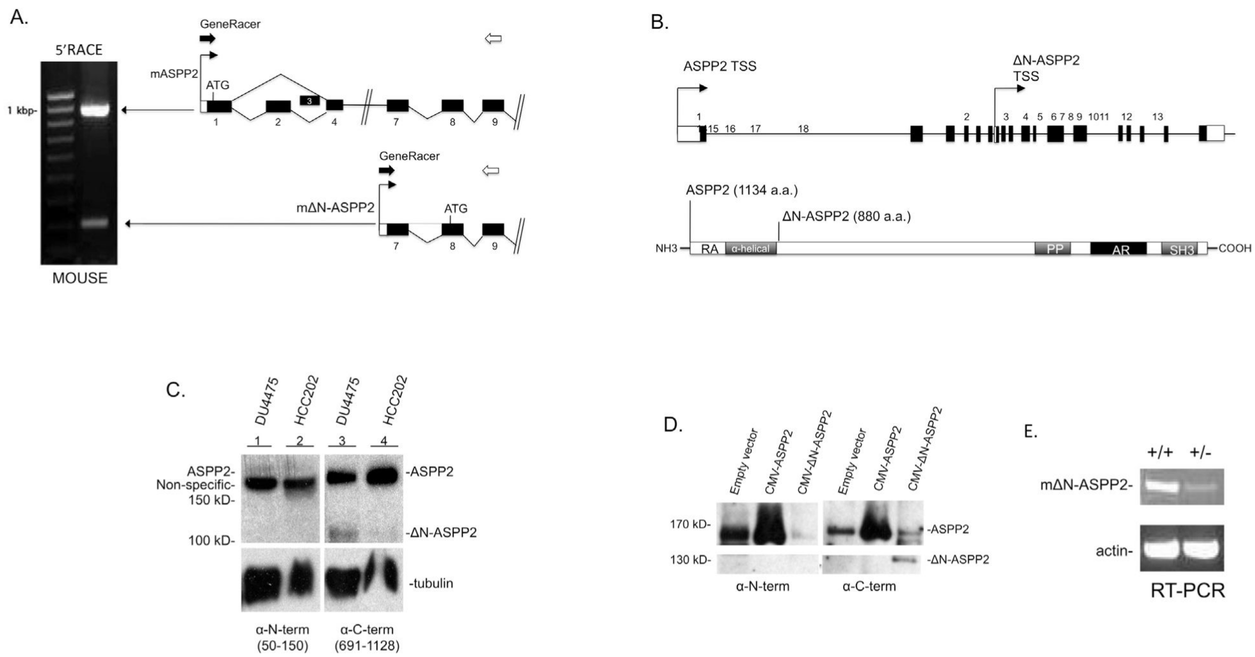


**Fig. 1. ΔN-ASPP2 is a novel N-terminally truncated isoform of ASPP2 and is expressed in cells**

(A) Mouse 5′RACE products. Forward primers (black arrows) and *ASPP2*-specific reverse primers (white arrow). (B) Diagram of ASPP2 and ΔN-ASPP2 gene structure (top) and protein structure (bottom). RA = Ras-association domain; PP = poly-proline region; AR = ankyrin repeats. (C) Immunoblot of breast cancer cells probed for endogenous ASPP2 and ΔN-ASPP2. (D) Immunoblot of ΔN-ASPP2 or ASPP2 transfected cells, probed with ASPP2 antibodies. (E) Semi-quantitative RT-PCR of *mΔN-ASPP2* in wild-type and *mASPP2*$^{exon\ 10–17/+}$ mice.

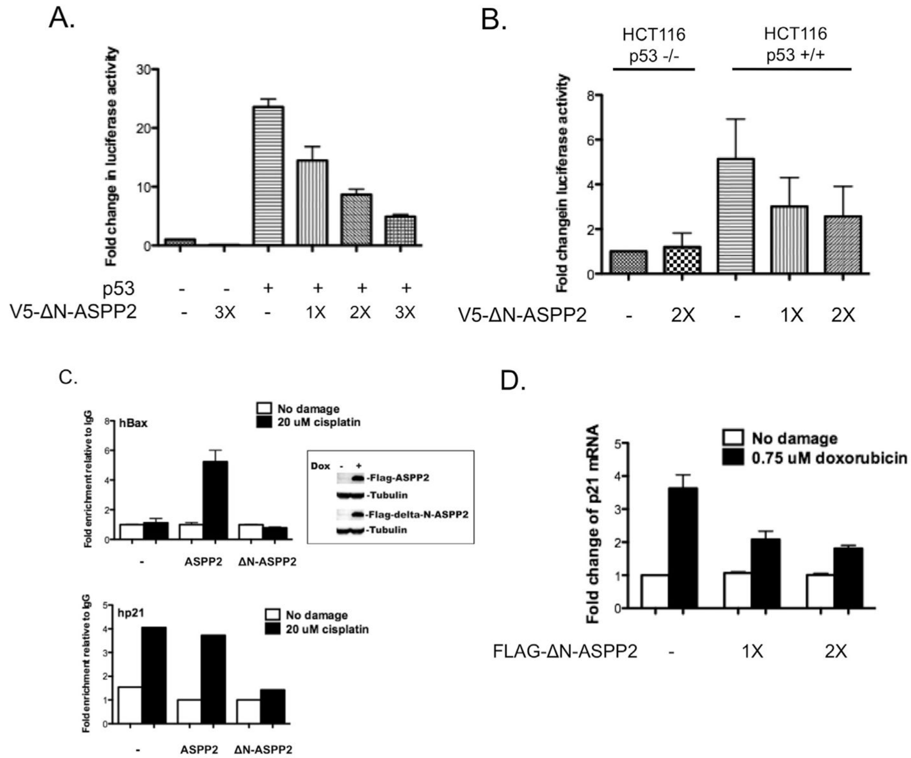


**Fig. 2. ΔN-ASPP2 inhibits p53 transcriptional activation**

(A) p21-luciferase activity in H1299 cells after transfection of indicated expression plasmids. (B) p21-luciferase activity in isogenic HCT116 cell lines after transfection with ΔN-ASPP2. (C) Chromatin immunoprecipitation of U2OS tetracycline-responsive (tr) FLAG-ΔN-ASPP2 or tr-FLAG-ASPP2 cells following exposure to cisplatin. Quantitative PCR for *Bax* (upper panel) and *p21* (lower panel). Samples were normalized to percent input and equivalently processed IgG controls. (Inset) Immunoblot prepared from U2OS tr-FLAG-ΔN-ASPP2 or FLAG-ASPP2 cells. (D) Quantitative RT-PCR of *p21* mRNA in HCT116p53$^{+/+}$ cells.

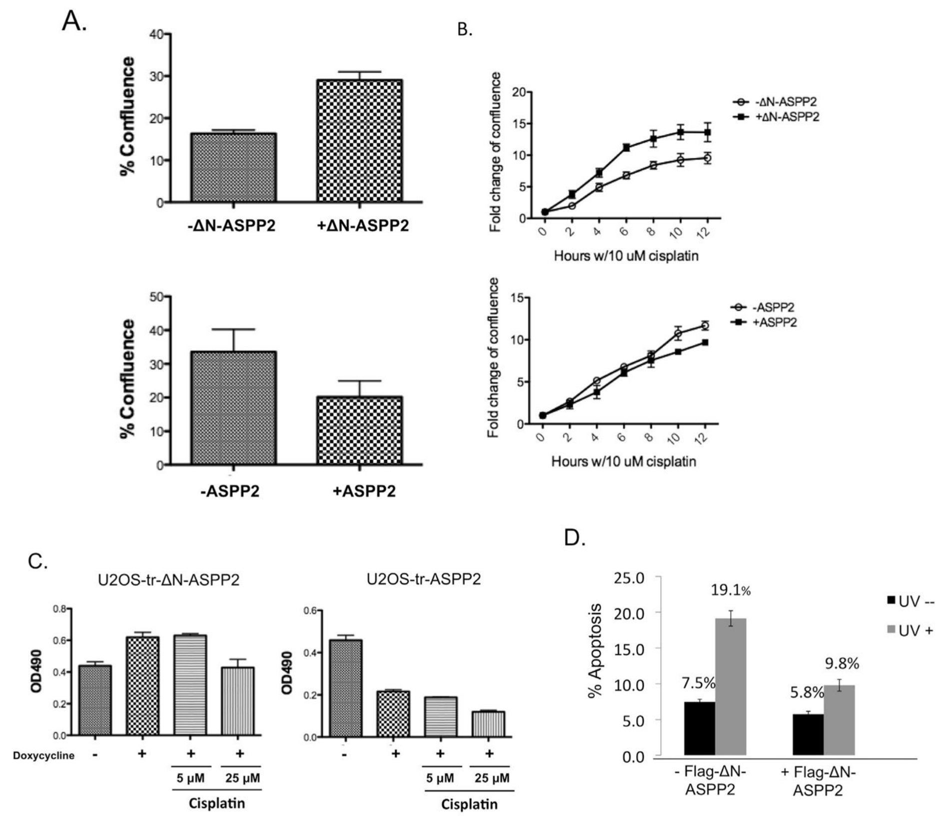


**Fig. 3. ΔN-ASPP2 enhances cell proliferation and survival**

(A) Percent confluence for U2OS cells with and without FLAG-ΔN-ASPP2 (top) or FLAG-ASPP2 (bottom). (B) Rate of confluence change for U2OS cells with or without FLAG-ΔN-ASPP2 (top) or FLAG-ASPP2 (bottom) following exposure to cisplatin. (C) MTS assay for tetracycline-regulatable U2OS cells with or without FLAG-ΔN-ASPP2, or with or without FLAG-ASPP2, following exposure to cisplatin. (D) Percent Annexin V positive cells in U2OS cells transfected with FLAG-ΔN-ASPP2 and exposure to 40 J/m$^2$ UVC.

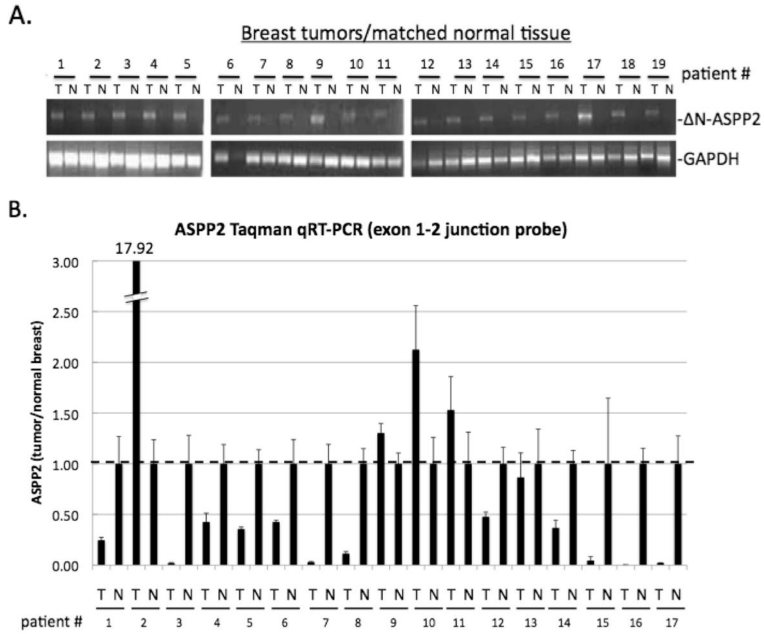


**Fig. 4. ΔN-ASPP2 is overexpressed in breast tumors**

(A) Semiquantitative *ΔN-ASPP2* in tumor (T) and adjacent normal tissue (N). (B) Quantitative RT-PCR of *ASPP2* in tumor (T) and adjacent normal tissue (N). *ΔN-ASPP2* and *ASPP2* expression in tumors is relative to matched normal tissue.