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ASB11在BIK泛素化與細胞凋亡調控中所扮演的角色

The role of ASB11 in BIK ubiquitination and apoptosis regulation

何其寰

Chi-Huan, Ho

指導教授：陳瑞華 博士

Advisor: Ruey-Hwa Chen, Ph.D.

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口試委員會審定書

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本論文係何其寰君（R03B46005）在國立臺灣大學生化科學研究所完成之碩士學位論文，於民國 105 年 7 月 29 日承下列考試委員審查通過及口試及格，特此證明。

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感謝與我共度過去兩年的師長、家人，以及同儕；

因為你們，這篇論文才得以誕生。

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中文摘要

BIK (Bcl-2-interacting killer)屬Bcl-2蛋白質家族中之BH3-only成員，且因其促細胞凋亡之性質而被認為是腫瘤抑制基因。然而，細胞內BIK蛋白表現量之調控機制至今尚未被完全了解。基於過表現ASB11 (ankyrin and SOCS box protein 11)可促進BIK泛素化之事實，本實驗室先前已鑑別出ASB11蛋白可能作為負責介導BIK泛素化之受質辨識單元(substrate recognition subunit, SRS)，參與在Cullin5 E3接合酶複合體(Cul5 E3 ligases complex)所催化之泛素化反應中。而在本次研究中，我們藉Co-IP以及in vitro binding assay驗證了ASB11與BIK之間所存在的直接性結合，並於in vitro ubiquitination assay中證明重組之ElonginBC-Cul5-ROC2-ASB11複合體具有催化BIK泛素化之酵素活性。進一步的實驗則顯示，過表現ASB11將會導致293T細胞株內之BIK蛋白含量下降；且此現象可藉由施以蛋白酶體抑制劑MG132所排除，指出ASB11可促使BIK蛋白經泛素-蛋白酶體系統(ubiquitin-proteasome system, UPS)被降解。與此相應，過表現ASB11亦可部分性地抑制由DNA損害藥物(DNA damage agents)處理所引發之細胞凋亡，顯示ASB11具有促進細胞存活之能力。另一方面，HCT116細胞株中ASB11之mRNA表現量被發現會在經過cisplatin, doxorubicin以及5-fu等DNA損害藥物之處理後經由p53路徑被負調控，顯示ASB11可能參與在DNA損害反應(DNA damage response)之中。此外，tunicamycin及thapsigargin等內質網壓力誘導物(ER-stress inducers)之處理則被發現可以透過XBP1路徑提高293T細胞株中*ASB11*之mRNA表現量，並且降低BIK蛋白之表現量；此結果暗示ASB11可能參與在不正常蛋白質摺疊反應(unfolded protein response, UPR)的適應性反應(adaptive response)之中。綜合以上結果，本研究發現了Cul5-ASB11所介導之BIK泛素化機制；並且指出此機制在抑制DNA損害藥物所引發之細胞凋亡，以及在針對內質網壓力之適應性反應中所扮演的角色。

關鍵字：BIK, ASB11, 細胞凋亡, 泛素-蛋白酶體系統, DNA損害, 內質網壓力

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Abstract

BIK (Bcl-2-interacting killer) is a BH3-only Bcl-2 family protein which has been considered as a tumor suppressor based on its pro-apoptotic function. However, the mechanism that regulates BIK protein level has not been completely understood. Previous studies in our laboratory identified ASB11 (ankyrin and SOCS box protein 11) as a candidate of the substrate recognition subunit (SRS) that recruits BIK to Cullin5 (Cul5) E3 ligases complex. In this study, we first examined the direct relationship between BIK and ASB11. Through co-IP and in vitro binding assay, the interaction between ASB11 and BIK was demonstrated. Furthermore, in vitro ubiquitination assay revealed the capacity of reconstituted ElonginBC-Cul5-ROC2-ASB11 complex to promote BIK polyubiquitination. Accordingly, overexpression of ASB11 in 293T cells decreases BIK protein abundance. Together with the finding that MG132 treatment blocks BIK downregulation induced by ASB11 overexpression, our data indicate that ASB11 promotes BIK degradation through ubiquitin-proteasome system (UPS). Consistent with the downregulation of pro-apoptotic BIK, ASB11 elicits a pro-survival effect to inhibit apoptosis induced by DNA damage agents. Furthermore, we show that DNA damage agents induced downregulation of *ASB11* mRNA in a p53-dependent manner. On the contrary, the ER-stress inducers tunicamycin and thapsigargin unregulated *ASB11* mRNA expression and reduced BIK protein abundance in 293T cells through a XBP1 dependent pathway. Together, this study identifies a BIK degradation pathway mediated by Cul5-ASB11 ubiquitin ligase complex and implies a role of this pathway in both antagonizing p53-dependent apoptosis in response to DNA damage agents and the adaptive response to ER stress.

Keyword: BIK, ASB11, apoptosis, UPS, DNA damage, ER stress

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I. Introduction

1. Apoptosis

Apoptosis is an evolutionary conserved mode of programmed cell death which results in suicide without cell lysis and activation of inflammatory response [1-3]. Apoptosis is distinguished from other forms of cell death by its distinct morphological features [4]. For instance, chromatin condensation and cell shrinkage occur during the early stage of apoptosis, followed by plasma membrane blebbing and DNA fragmentation [4]. The cellular constituents are subsequently packaged into blebs and separated from the cell to form apoptotic bodies. The compact, membrane-enclosed apoptotic bodies containing all the cellar fragment are then removed by phagocytes through the phagocytic process [3].

Apoptosis can be triggered by extrinsic or intrinsic stimuli by different signaling pathways, but both extrinsic and intrinsic pathways converge onto the same terminal step called caspase cascade [5, 6]. Caspases (cysteine-dependent aspartate-directed proteases) specifically cleave substrates at the peptide bonds after an aspartate residue, through a cysteine-dependent reaction mechanism. Caspases can be classified into different types according to their roles in different biological processes. Among them, initiators (caspase-2,-8,-9,-10) and executioners (caspase-3,-6,-7) play the central roles in apoptosis machinery. Since caspases are synthesized as inactive proenzyme called procaspases, they have to be proteolytically cleaved at specific sites to gain their enzymatic function. Initiators are first activated in the apoptosis processes, and the activated initiators can in turn cleave and activate downstream executioners. Once

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executioners are activated, various cellular substrates are cleaved to result in morphological change and cell death [7].

The extrinsic pathways are responsible for extracellular signals, such as FAS ligand and TNF [8]. Upon the binding of these extracellular factors to their cognate receptors, such as FAS/CD95 and TNFR (also known as death receptors), cytoplasmic adaptor proteins are recruited to the death receptor [8]. This leads to the further recruitment of initiator caspases-8 and ceaspase-10. The receptors, adaptors and initiator caspases form large multimeric complex called death-inducing signal complex (DISC), where initiator caspases are activated to result in the activation of executioners and cell death [3, 9].

In contrast to extrinsic pathways, the intrinsic pathways are triggered by various intracellular stimuli which result in mitochondrial permeabilization and the release of proapoptotic molecules such as cytochrome c, Smac/DIABLO, and HtrA2/Omi. Once in the cytoplasm, cytochrome c binds to Apaf-1 and procaspase-9 to from a multimeric apoptosome complex, which induces the self-processing of procaspase-9 to make the active caspase-9. Caspase 9 subsequently activates executioner caspase-3 by cleaveing procaspase-3. In addition to cytochrome c, the released Smac/DIABLO interacts with the inhibitors of apoptosis proteins (IAPs), which interact and inhibit the caspases to suppress apoptosis, and then antagonizes IAPs to induced apoptosis [10]. In the other hand, the HtrA2/Omi, which is a member of the HtrA serine protease family, induces apoptosis by promoting the cleavage and degradation of the IAPs [11]. The activation of intrinsic pathway is tightly regulated by the Bcl-2 protein family, which comprise pro-apoptotic as well as anti-apoptotic members [3, 9].

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2. Bcl-2 protein family

Bcl-2 family proteins are characterized by their Bcl-2 homology (BH1-4) domains, which are responsible for the critical homo- and hetero- dimerization ability of Bcl-2 family proteins. Bcl-2 family proteins are involved in various biochemical processes, such as calcium homeostasis, mitochondrial morphology regulation, cell cycle checkpoint, glucose metabolism, ER-stress response, and most importantly, apoptosis [12, 13]. According to their structure and roles in apoptosis regulation, Bcl-2 family members could be classified into three groups: anti-apoptotic, multi-domain pro-apoptotic, and BH3-only pro-apoptotic members.

All of the reported anti-apoptotic Bcl-2 family proteins, include Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1, have multiple BH domains. The BH1-3 domains construct a hydrophobic surface groove with a central hydrophobic α-helix that interacts with the hydrophobic region of the amphipathic α-helix formed by BH3 domain of a pro-apoptotic family member. In this way, the anti-apoptotic Bcl-2 family proteins heterodimerize with pro-apoptotic members and inhibit the pro-apoptosis members to promote cell survival. The BH4 domain of anti-apoptotic members can further stabilize the structure formed by BH1-3. Loss of BH4 domain impairs the dimerization and the anti-apoptosis ability of anti-apoptosis members. Removal of BH4 through cleavage by caspase switches Bcl-2 and Bcl-xL from anti-apoptotic into pro-apoptosis proteins [14-16].

The multi-domain pro-apoptotic Bcl-2 family proteins are known as “effectors”. Two of the members, BAX and BAK, undergo conformational change during the initiation of apoptosis to form the pore-like homo-oligomer complex on the

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mitochondrial outer membrane. This event further induces the mitochondrial outer membrane permeabilization and the release of pro-apoptotic molecules [17].

The BH3-only proteins are the Bcl-2 family members that do not contain BH1, BH2 and BH4 domains, but they can still form dimers with other Bcl-2 family proteins thought their BH3 domain. BH3-only proteins alter the dynamic balance between pro- and anti-apoptotic members and promote apoptosis by specifically inhibiting the anti-apoptotic family members or directly activating the multi-domain pro-apoptotic members. BH3-only proteins are induced by transcriptional or post-translational mechanism in response to various stress signals and play critical roles in the regulation of apoptosis and the homeostasis of Bcl-2 family proteins [17].

The Bcl-2 family members function as the checkpoint in apoptosis process. Changes in the regulation of Bcl-2 family proteins alter the fate of cells. For instance, p53 transcriptionally upregulates BIK, NOXA, PUMA and BAX in the DNA damage response to contribute to apoptosis [18-21]. In additional, the JNK-promoted phosphorylation induces apoptosis by activating BAD, BID, BIM and BAX as well as inhibiting Bcl-2, BcL-xL and Mcl-1 in various stress conditions [22-28]. On the contrary, the cytokine-induced protein kinase ERK1/2 promote cell survival by phosphorylating Bcl-2, Mcl-1, BIM, BIK, and BAX. Phosphorylation by ERK1/2 can inhibit BAX activity, stabilize Mcl-1, and promote BIM and BIK degradation through ubiquitination-proteasomal pathway, and is required for the anti-apoptotic activity of Bcl-2 [29-35]. Ubiquitin-proteasome pathway plays an important role in the regulation of Bcl-2 family members. For example, BIM can be ubiquitinated and targeted for proteasomal degradation by TRIM2, βTrCP, and c-Cbl [36-38]. Interestingly, βTrCP

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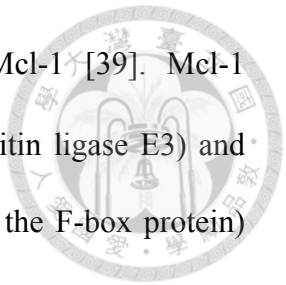

also promoted ubiquitin-mediated proteasomal degradation of Mcl-1 [39]. Mcl-1 ubiquitination can also be promoted by βTrCP, Mule (Mcl-1 ubiquitin ligase E3) and SCF^{FBW7} (the SKP1–cullin-1–F-box complex which take FBW7 as the F-box protein) [40, 41].

2.1. BIK

BIK (BCL-2 interacting killer), the first member of the pro-apoptotic BH3-only proteins, is a 18 kDa, 160 amino acids protein which contains a BH3 domain and a transmembrane domain (TM) [42, 43]. The BH3 domain is required for the pro-apoptotic function of BIK, while the TM is required for anchoring BIK to endoplasmic reticulum membrane but not for apoptosis induction [44]. BIK is able to interact with mammalian and viral anti-apoptotic proteins, including BCL-2, BCL-xL, Mcl-1, adenovirus E1B-19K, and EBV-BHRF1 [42, 45]. The interaction between BIK and anti-apoptotic proteins liberates Bax/Bak and promotes apoptosis [45]. The efficient pro-apoptotic activity of BIK is dependent on the phosphorylation of BIK on Thr33 and Ser35. The Thr33A/Ser35A mutant, which cannot be phosphorylated, has a lower pro-apoptosis activity than the wild-type BIK [46]. On the other hand, the phosphorylation-mimicking Thr33D/Ser35D mutation enhances the ability of BIK to interact with Bcl-2 and BCL-xL, as well as the pro-apoptotic activity of BIK [47].

BAX/BAK are considered to be redundant for the death-promoting function of various BH3-only proteins including NOXA, BIM and BID, because apoptosis induced by those BH3-only proteins can only be abolished by BAX/BAK double deficiency but not by single deficiency [48, 49]. Interestingly, it was found that pro-apoptotic ability of BIK is only dependent on BAX but not BAK in several human cell lines [50-52].

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Although the study in 293T cells showed that BIK is able to interact with Mcl-1 efficiently and results in BAK releasing and apoptosis activation, the studies in BAX-deficient HCT116 and DU145 cells, in which BIK fails to induce apoptosis, purposed a different view [45, 50, 51]. In such cell systems, BIK cannot interact with Mcl-1, suggesting that BIK only has limited affinity to Mcl-1 [52]. Additionally, Mcl-1 knockdown sensitizes BAX-deficient DU145 cells to BIK, suggesting that BIK can still activate BAK by liberating them from BCL-xL. According to those results, it is suggested that Mcl-1 may play a role in BIK inhibition by sequestering BAK that has just released from BCL-xL by BIK [52, 53].

Similar to other BH3-only pro-apoptotic proteins, BIK can be induced by various stress stimuli. Since BIK is a transcriptionally target of p53 and E2F, it can be upregulated by the accumulation of p53 and E2F induced by adenovirus infection and the expression of E1A [21, 54, 55]. DNA damage agents and radiation treatment can also induce BIK expression through p53-dependent manners [56]. In addition to p53 and E2F, BIK is directly activated by the transcription factor Smad 3/4 complex, which mediates the pro-apoptotic signal induce by TGF-β in a B-lymphoma cells line [57].

BIK is considered as a tumor suppressor, since BIK deficiency is found in several cancer types. For instance, deletion of chromosome region that contains BIK has found in clear-cell renal cell carcinoma (RCC) [58], glial brain [59], colorectal cancer [60], and oral cancer [61]. Mutation of BIK gene occurs frequently in the human peripheral B-cell lymphomas [62], and the loss of BIK accelerates murine lymphoma development [63]. Additionally, BIK is required for cell death induced by estrogen starvation and antiestrogen fulvestrant treatment in human breast cancer cells [56, 64], while the high

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expression of the GRP78, an ER chaperone functioning as BIK inhibitor and the major ER-stress sensor, correlates with metastasis, malignancy, and drug resistance of human breast cancer [65-69] .

3. The Ubiquitin-Proteasome System

Protein homeostasis is important for proper cellular function, thus both protein synthesis and degradation need to be tightly regulated. The ubiquitin-proteasome system (UPS) represents a critical mechanism to degrade the majority of cellular proteins in eukaryotes. Proteins that undergo UPS would first be modified by ubiquitination then be recognized and proteolyzed by the 26S proteasome [70].

Ubiquitination is the post-translational modification that covalently attaches the ubiquitin, an evolutionarily conserved 76 amino acids protein, to a substrate through a isopeptide bond [70]. Typically, the isopeptide bond is forming between the C-terminal glycine of ubiquitin and a lysine residue on the substrate, but it is reported that ubiquitination can also happen on other residues of substrates [70, 71]. The complexity of ubiquitination topologies provides the potential for ubiquitination to encode diverse signals for determining the fate of modified substrates, which includes but not limited to proteasomal degradation. Adding one ubiquitin to a single site is referred to as monoubiquitination, which can occurs multiple times at different sites of a substrate and then form the multimonoubiquitination. A ubiquitinated substrate can further be polyubiquitinated, by adding a new ubiquitin to the lysines (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) or Met-1 residues of the ubiquitins that already attached [72]. Polyubiquitination is classified into different chain types. When all the ubiquitins attached on the polyubiquitinated substrate are modified on the same site, the chain is

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defined as a homogeneous ubiquitin chain. By contrast, a chain is defined as the mixed ubiquitin chain if ubiquitin molecules within the chain are modified at different sites. In addition, a single ubiquitin molecule can also be modified at more than one sites thereby forming branched ubiquitin chain [73]. The Lys48-linked polyubiquitin chain is the classical chain type that plays the major role in the UPS, though other chain types are also involved [73, 74].

3.1. The E3 ubiquitin ligases

The canonical ubiquitination is dependent on sequentially catalyzed by the ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligating enzyme (E3). The E1 consumes one molecule of ATP to store the energy in the thioester bond forming between the ubiquitin and the active-site cysteine of the E1 during the activation of ubiquitin, and then the ubiquitin is transferred to the active-site cysteine of the E2 to form the charged E2~Ub. Finally, the E3 ligase, which plays the key role in substrate recognition, transfers ubiquitin from the E2~Ub to the substrate [73].

The E3 ligase is categorized into two major types, the RING (really interesting new gene) family and the HECT (homologous to E6AP C terminus) family, which catalyze ubiquitination in distinct mechanisms. Members of the HECT members receive Ub from E2~Ub and form the E3~Ub intermediate and then transfer Ub from E3~Ub to substrates, whereas the RING finger-containing family transfers the ubiquitin directly from the E2~Ub to the substrate.

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3.2. The Cullin-RING E3 ligases

The Cullin-RING ligases (CRL) complex is the largest known class of E3 ligases. A Cullin-RING ligase complex is composed of a Cullin (Cul1, 2, 3, 4A, 4B, 5, 6, 7, 9, or APC2), a RING H2 finger protein, and a substrate-recognition subunit (SRS). Additionally, for some subtypes of CRLs, adaptors are required for anchoring the SRS to the Cullin complex.

In a CRL complex, the Cullin acts as the scaffold which interacts with the SRS and the adaptor through the N-terminal domain while the C-terminal domain binds to the RING H2 finger protein. The RING H2 finger protein is the catalytic subunit, which transfers ubiquitin from E2~Ub to the substrate. The SRS is responsible for the substrate binding and specificity of the E3. [75-77]

3.3. The ASB11

ASB11 belongs to the ankyrin and SOCS box (ASB) family, which is one of the member of the SOCS (suppressor of cytokine signaling) box-containing protein family [78]. The SOCS box-containing proteins function as the SRSs of the Elongin-Cullin-SOCS (ECS) E3 ubiquitin ligases complexes, a subtype of Cullin-RING E3 ligases. An ECS complex consists of elongin B, elongin C, Cul5, RBX2/ROC2 and SOCS box-containing protein. The SOCS box-containing proteins interact with the elongin B/C heterodimer and the Cul5 through their C-terminal SOCS box domains, while the substrates are recognized by their diverse N-terminal domains [79].

ASB11 is a endoplasmic reticulum resident protein, which interacts with substrates through its' ankyrin repeats domain and recruits substrates to the E3 complex

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to promote ubiquitination [78]. It was reported that *Danio rerio* version of ASB11 (d-ASB11) mediates the degradation of Delta A, the Notch ligand, and positive regulates Notch signaling [80]. Furthermore, ASB11 is required for the regulation of the size of the neural progenitor compartment and regenerative myogenesis in zebra fish [80, 81].

4. Endoplasmic reticulum stress

In the endomembrane system of eukaryote, the endoplasmic reticulum (ER) plays the key roles in lipid synthesis, cellular calcium homeostasis and protein processing [82]. To ensure the proper folding of proteins, the ER lumen is rich in molecular chaperones and folding enzymes, such as the GRP78 (glucose-regulated protein, 78 kDa) and the protein disulphide isomerase (PDI), respectively [83]. Disturbing ER protein homeostasis results in the accumulation of unfolded proteins, which is referred to as ER stress, which then induces the unfolded protein response (UPR). During the early phase of UPR, the upregulation of chaperone expression, the suppression of global translation, and the ER-associated protein degradation (ERAD) coordinate to restore ER protein homeostasis. However, once UPR fails to eliminate the stress condition, it will lead to apoptosis [82, 83].

The ER stress sensor GRP78, inositol-requiring enzyme 1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6) play the central roles in the UPR initiation. IRE1, PERK and ATF6 are ER transmembrane proteins that are inhibited by binding to ER chaperone GRP78 in the normal condition. Under ER stress condition, accumulated unfolded proteins occupy the GRP78, thereby liberating IRE1, PERK, and ATF6 to initiate UPR [82, 83].

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IRE1 is a bifunctional transmembrane kinase/endoribonuclease which is activated through oligomerization and trans-autophosphorylation after released from GRP78 [84]. The RNase domain of IRE1 catalyzes the non-conventional splicing of XBP1u (X-box binding protein 1, un-spliced form) mRNA to form the XBP1s (X-box binding protein 1, un-spliced form) mRNA [85-87]. The XBP1s protein is a transcription factor that promotes the expression of both pro-adaptive and pro-apoptosis UPR-related genes, including chaperones, ER degradation-enhancing α-mannosidase-like protein (EDEM), and the pro-apoptotic transcription factor C/EBP homologous protein (CHOP) [88, 89]. Besides, IRE1 influences protein homeostasis by promoting cleavage and degradation of various RNAs, such as 28S rRNA, through the a process called IRE1-dependant decay of mRNA (RIDD) [90].

PERK is a Ser/Thr protein kinase, which is activated after released from GRP78 by oligomerization and trans-autophosphorylation. Active PERK then phosphorylates the eukaryotic initiation factor-2α (eIF2α) to suppress global translation, thereby relieving ER stress [91]. Despite the attenuation of general translation, the translation of activating transcription factor 4 (ATF4) is selectively enhanced by eIF2α phosphorylation [92, 93]. ATF4 promotes the expression of various pro-survival genes which are involved in protein folding, amino acid import/metabolism, glutathione biosynthesis, and oxidative stress resistance [94, 95]. The directly targets of ATF4 also include the pro-apoptotic proteins such as CHOP, which is responsible for the pro-apoptotic function of PERK-ATF4 axis [96].

ATF6 is a bZIP family transcription factor, which is translocated to Golgi after released from GRP78. In the Golgi, ATF6 is sequentially cleaved by site-1 protease

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(S1P) and site-2 protease (S2P) to remove its luminal domain and transmembrane domain, respectively [97, 98]. The cleavage process activates ATF6 and allows it to translocate to the nucleus and upregulates the expression of various genes involving in protein folding, quality control and ERAD, such as GRP78 and PDI [99]. Similar to IRE1 and PERK, ATF6 also promotes apoptosis through CHOP [96].

4.1. ER stress in the live/death decision

Although the IRE1, PERK, and ATF6 pathways promote the protein refolding and cell survival in distinct mechanisms, all of them converge to the CHOP pathway to promote cell death if the accumulation of unfolded proteins reach to an excessive level and the cell stress becomes irreversible. CHOP suppresses the expression of pro-survival proteins such as Bcl-2 and induces the expression of pro-apoptotic BIM, BAX, PUMA, caspase-3, GADD34 (growth arrest and DNA damage-inducible protein), and endoplasmic reticulum oxidoreductin-1α (ERO1α) to promote cell death [100]. The GADD34 forms complex with the protein phosphatase-1 (PP1) to dephosphorylate eIF2α and restores the translation machinery. On the other hand, the ERO1α produces the reactive oxygen species (ROS) and increases the oxidative stress of ER then activates the ER calcium channel inositol 1,4,5-trisphosphate receptor type 1 (IP3R1), resulting in the release of calcium to cytosol. Increasing in cytosol ROS and calcium then induces the permeabilization of mitochondria and releasing of cytochrome c, which promote apoptosis.

Besides CHOP pathway, IRE1 can also promote apoptosis by forming complex with the TNF receptor-associated factor 2 (TRAF2), which phosphorylates the apoptosis signal-regulating kinase 1 (ASK1) and activates the pro-apoptotic c-Jun N-

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terminal kinase (JNK) pathway [90, 101]. Furthermore, it was reported that active ATF6 is able to upregulate the expression of pro-apoptotic WBP1 (WW domain binding protein 1), which promotes apoptosis through downregulating the cellular abundance of Mcl-1 [102].

4.2. Bcl-2 family proteins in UPR regulation

In addition to the regulation of mitochondrial apoptotic pathway, Bcl-2 family members play crucial roles in regulating ER stress response. For example, BAX and BAK directly interact with IRE1 to promote the activation of IRE1 [103]. Compared to control mice, the BAX and BAK double knockout mice represent poorer ability to induce XBP-1s and JNK phosphorylation in response to tunicamycin-induced ER stress. BIM and PUMA also directly interact with IRE1 while the BIM and PUMA double knockout facilitates the inactivation of IRE1, indicating that BIM and PUMA are required for maintenance the activity of IRE1 [104].

Bcl-2 family members are also involved in the regulation of ER calcium homeostasis and apoptosis induced by calcium efflux from the ER. For instance, Bcl-2 inhibits the calcium efflux by inhibiting IP3R1 and suppressing the calcium-induced apoptosis. Bcl-2 also decreases the calcium concentration in the ER by destabilizing the ER calcium transporter, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) [105]. By contrast, BAX and BAK are required for the maintenance of high calcium concentration in ER and for the activation of IP3R1 [106, 107].

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5. DNA damage response

The chemical structure of DNA can be damaged by various physical and chemical agents, or by errors in DNA replication. Since damage to the genomic DNA can result in mutations and the development of various diseases including cancer, repair mechanisms are required for maintenance of genomic stability. On the other hand, if the repair processes fail to restore the gene integrity, the apoptosis machinery is required to maintain health of the individual by eliminating damaged cells.

Various kinds of DNA damages have been reported, including single-strand break (SSB), double-strand break (DSB), DNA adducts, mismatching and the modifications at nucleotide residues. Different kinds of damage would trigger distinct pathways of DNA-damage response and results in different cell fates.

5.1. Apoptosis in DNA-damage response

To ensure the execution of the cells which are unable to properly repair their damaged DNA, the DNA-damage response facilitates apoptosis through various pathways. For instance, the Mre11-Rad50-Nbs1 complex recognizes the DSB and activates the ATM (ataxia telangiectasia mutated), a serine/threonine protein kinase [108-110]. The substrates of ATM includes various proteins involving in DNA-damage repair, cell cycle arrest and apoptosis, such as checkpoint homolog 1/2 (Chk1/2), breast cancer 1 (BRCA1), and p53 [111]. On the other hand, the replication protein A (RPA) and the ATR-interacting protein (ATRIP) recruit another important protein kinase ATR (ATM and Rad3 related) to the single-stranded DNA during the repair or replication of damaged DNA [112-114]. The ATR then modulates DNA repair, cell cycle checkpoint

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and apoptosis by phosphorylating its' substrates, which also includes p53 and Chk1 [115, 116].

p53, which is stabilized by ATM/ATR mediated phosphorylation, plays a critical role of tumor suppressor. In addition to cell cycle arrest and DNA-damage repair, p53 upregulates a set of proteins that are involved in apoptosis, such as BAX, PUMA and p21 [18, 20, 117]. On the other hand, the phosphorylated Chk1/2 promotes apoptosis in a p53-independent manner. By activating the transcription factor E2F1, Chk1/2 indirectly increases the expression of p73, which then induces the expression of PUMA, NOXA, BAX and BIK to promote apoptosis [118-121].

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II. Materials and Methods

Plasmids

The plasmid encoded ubiquitin was described previously [122]. The plasmids encoded HA-Elongin B, T7-Elongin C and HA-ROC2 were purchased from Addgene. Elongin B and ROC2 were then cloned into pRK5-HA and pRK5-V5 vector respectively. Other plasmids used for the transient transfection were established in our lab previously by inserting Cul5, BIK, and ASB11 genes into pRK5 vectors with indicated tags.

The pLAS5w.Pneo-p53 and pLAS5w.Pneo-ASB11 plasmids were generated in our lab and were used to stable overexposes indicated genes in cultured cell lines by the lentiviruses system. The plasmid encoded p53 was a kindly gift from Dr. Shieh, Sheau-Yann and then the p53 gene was subcloned into the pLAS5w.Pneo vector. The pLAS5w.Pneo vector and shRNA-expressing constructs were obtained from National RNAi Core Facility, Taiwan. The Clone ID of shRNA-expressing construct are:

shASB11 : TRCN0000164096

shXBP1#2 : TRCN0000277990

shXBP1#3 : TRCN0000278051

Cell culture and transient transfection

293T, 293FT, and H1299 cells were cultured in Dulbecco's modified Eagle Medium (DMEM) containing 10% fetal bovine serine (FBS) and 1% penicillin/ streptomycin (PS), whereas HCT116 cells were cultured in RPMI1640 Medium

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containing 25mM HEPES, 10% fetal bovine serine (FBS) and 1% penicillin/ streptomycin (PS). All the cells were maintained in the 5% CO2, 37°C humidified condition. 293T and 293FT were transfected by the calcium-phosphate method, whereas the transfection of HCT116 was performed by lipofectamine 3000 reagent that purchased from Thermo Fisher Scientific.

Lentivirus and infection

Lentiviruses were generated by transient transfection 293FT cells with packing plasmid (pCMVΔ8.91), envelope VSV-G plasmid (pMD.G) and shRNA clones or overexpression vectors. For a 10 cm^2 dish, 14 μg pCMVΔ8.91 plasmid, 2 μg pMD.G plasmid, and 14 μg shRNA clones or overexpression vectors were used. 8 hours after transfection, the medium was refreshed with 6 ml culture medium. For 42~48 hours harvest, supernatants were filtered by 0.45 μm pore-size syringe filter. The virus-containing mediums were added into cells with 8 μg/ml polybrene. One day after infection, the mediums were refreshed with culture mediums. The selection of infected cells were applied 2 day after infection, by specific antibiotics.

Antibodies and reagents

Mouse anti-Flag (M2; Sigma), mouse anti-His (Santa Cruz), mouse anti-Tubulin (Millipore), mouse anti-Myc (invitrogen), mouse anti-HA (Sigma), goat anti-BIK (N-19; Santa Cruz), mouse anti-V5 (Millipore) and mouse anti-p53 (Santa Cruz) antibodies were purchased from commercial sources. The ASB11 antibodies was provides by LTK BioLaboratories, and the synthetic peptide (CTDYGANLKRRNAQGKSAL) was used as the antigen to generated antibodies.

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The MG132 (Calbiochem), cycloheximide (Sigma), thapsigargin (Cayman Chemical), tunicamycin (Cayman Chemical), doxorubicin (Tocris Bioscience), cisplatin (Sigma), 5-fu (Sigma), etoposide (Sigma) were also purchased from commercial sources.

Cell lysates preparation

Cells were lysed by 1X RIPA buffer (150 mM NaCl, 20 mM Tris-HCl [pH 7.5], 0.1% SDS, 1% sodium deoxycholate and 1% NP40) with protease inhibitors (10 mg/ml aprotinin, 1 mM PMSF and 10 mg/ml leupeptin). Cell lysates were sonicated and centrifuged, the supernatants were subject to protein quantification by Bradford reagent (Bio-Rad, Hercules, CA).

Western blotting

Sample were prepared by mixed cell lysates with the sample buffer (10% glycerol, 50 mM Tris-HCl [pH 6.8], 2% SDS, 0.01% bromophenol blue and 8% β-mercaptoethanol) and incubated at 95°C for 5 min. Protein samples were resolved by SDS-PAGE according to standard protocol and transferred onto PVDF membranes. The membranes were blocked in the blocking buffer (Tris buffered Saline with 0.1% Tween-20 and 3% skin milk) at room temperature for at least 30 min and then incubated overnight in the blocking solution with diluted primary antibodies, at 4°C. Next, the PVDF membranes were washed in 0.1% TBST (Tris buffered Saline with 0.1% Tween-20) three times for 10 min each. PVDF were then incubated in the blocking solution with indicated HRP-conjugated-secondary antibodies for 1 hour at room

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temperature. Finally, the membranes were washed in 0.1% TBST three times for 20 min each before the ECL (Amersham) detection.

Immunoprecipitation

The 293T cells was transfected with pRK5-Flag-ASB11 plasmid 2 day before harvesting. 18 hours after transfection, the medium was refreshed with 7 ml culture medium, and then the cell was treated with MG132 (1 μM/ml) 24 hours after transfection. Cell lysates were incubated with anti-ASB11 agarose beads (Sigma) in 1X RIPA buffer at 4°C for 1.5 hr. The precipitates were washed with 1X RIPA for 4 times, then incubated in the sample buffer at 95 °C for 5 min. Finally, the precipitated proteins were detected by western blotting.

In vitro binding assay

Myc-ASB11 and 3XFlag-BIK proteins were generated by respectively transient transfecting 293T cells with the pRK5-Myc-ASB11 and pRK5-3XFlag-BIK plasmids 2 day before harvesting. 18 hours after transfection, the medium was refreshed with 7 ml culture medium, and then the cells were treated with MG132 (1 μM/ml) 24 hours after transfection. Myc-ASB11 proteins was immobilized by incubating cell lysates with anti-Myc agarose beads (Sigma) in 1X RIPA buffer at 4°C for 1.5 hr. The M2 beads were washed with 1X RIPA for 7 times. 3XFlag-BIK proteins was purified by incubating cell lysates with anti-Flag agarose beads (Sigma) in 1X RIPA buffer at 4°C for 1.5 hr. The Myc beads were washed with 1X RIPA for 7 times. The 3XFlag-BIK proteins were then eluted by incubated the beads in the elution buffer [50 mM Tris-HCl, pH 7.4, with 150 mM NaCl and 100 mg/ml 3XFLAG peptide (Sigma)] at 4°C for 2 hr.

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The immobilized Myc-ASB11 proteins were incubated in the 1X RIPA buffer with or without 3XFlag-BIK proteins at 4°C for 1.5 hr, and then was washed with 1X RIPA for 7 times. Finally, the immobilized proteins were incubated in the sample buffer at 95 °C for 5 min and were detected by western blotting.

In vitro ubiquitination assay

The reconstituted ElonginBC-Cul5-ROC2-ASB11 E3 complex were generated by co-transfecting 293T cells with the plasmids encoded each subunit 2 day before harvesting. 18 hours after transfection, the medium was refreshed with 7 ml culture medium, and then the cells were treated with MG132 (1 μM/ml) 24 hours after transfection. The complex was isolated by incubating cell lysates with anti-Flag agarose beads (Sigma) in 1X RIPA buffer at 4°C for 1.5 hr. The immobilized Myc-ASB11 protein was washed with 1X RIPA for 5 times. The 3XFlag-BIK proteins were generated by the same method as the in vitro binding assay. The assay was then be operated by incubated the reaction buffer (described previously [123]) with or without E1, E2, His-ubiquitin, reconstituted complex and 3XFlag-BIK proteins at 37°C for 2 hr 45 min. The E1, E2, His-ubiquitin and other related reagents used in this in vitro ubiquitination assay were purchased from R&D Systems.

In vivo ubiquitination assay

The plasmids encode His-ubiquitin and BIK were co-transfected into the HCT116 cells 2 day before harvesting. 6 hours after transfection, the medium was refreshed with 7 ml culture medium, and then the cell was treated with MG132 (1 μM/ml) 24 hours after transfection. Cell lysates were incubated with Ni-NTA sepharose beads (GE Healthcare Life Sciences) in 1X RIPA buffer at 4°C for 1.5 hr. The

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precipitates were washed with 1X RIPA for 4 times, then incubated in the sample buffer at 95 °C for 5 min. Finally, the precipitated proteins were detected by western blotting.

RNA extraction, reverse transcription, and real-time PCR

Total RNA of indicated cells were extracted by TRIZOL reagent (Invitrogene) through the standard protocol. The iScript™ cDNA Synthesis Kit (Bio-Rad) was used to reverse transcript the total RNA into cDNA. The cDNA were then quantified by real-time PCR, using the Roche LightCycler 480 system. The primers used for real-time PCR are:

GAPDH:	Forward	: 5' TGTTGCCATCAATGACCCCTT 3'
	Reversed	: 5' CTCCACGACGTACTCAGCG 3'
ASB11:	Forward	: 5' CCTGCTAACCGACTATGGAGC 3'
	Reversed	: 5' TAGGAGGAATCGTTCGAGTGG 3'
XBP1 :	Forward	: 5' CCCTCCAGAACATCTCCCCAT 3'
	Reversed	: 5' ACATGACTGGGTCCAAGTTGT 3'

PI staining and flow cytometry

Harvested cells were fixed by methanol and incubated at 4°C for at least one day. Fixed cells were washed with 1X PBS, and were stained by the propidium iodide. Flow cytometry experiments were operated on the BD FACSCalibur, and the CellQuest Pro software were used for analysis.

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III. Results

Cul5-ASB11 E3 complex mediates BIK ubiquitination

BIK is a labile protein, but its abundance can be drastically accumulated and stability can be greatly enhanced after treatment of cells with proteasome inhibitor bortezomib or MG132 [124]. This implies an important role of ubiquitin-proteasome system in BIK proteolysis. However, ubiquitin E3 ligase that can catalyze BIK ubiquitination is still unknown. Previous study in our lab identified Cul5-ASB11 E3 complex as a candidate ubiquitin ligase responsible for BIK ubiquitination, based on the fact that BIK ubiquitination was enhanced by ASB11 overexpression in 293T cells (Fei-Yun Chen, unpublished results).

Thus, we purposed a hypothesis that BIK is a direct substrate of Cul5-ASB11 E3 complex. To test our hypothesis, we first examined the interaction between BIK and ASB11 by employing a co-immunoprecipitation (co-IP) analysis. In this experiment, Flag-ASB11 was transfected into 293T cells, and cell lysate was used to perform the co-IP with anti-Flag M2 beads. We found that endogenous BIK was specifically coprecipitated with Flag-ASB11 (Fig. 1), which supports our hypothesis. Next, the interaction between ASB11 and BIK was confirm by the *in vitro* binding assay, in which the purified Myc-ASB11 was immobilized on anti-Myc beads and co-incubated with 3xFlag-BIK purified from transfected cells (Fig. 2). Thus, the *in vitro* and *in vivo* interaction assays indicate BIK as a binding partner of ASB11.

To further confirm that BIK is a direct substrate of ASB11-based ubiquitin ligase, we performed an in vitro ubiquitination assay. The ROC2/Cul5/Elongin B/C/ ASB11 E3 ligase complex purified from cotransfected cells was capable of promoting

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polyubiquitination of BIK in an E1/E2 dependent manner (Fig. 3). Together, our results indicate that BIK is a substrate of this E3 ligase complex.

ASB11 facilitates BIK degradation through ubiquitin-proteasome pathway

Following the discovery of ASB11-mediated BIK ubiquitination, we turned our interest into the biochemical and physiological significant of this ubiquitination. To explore the role of ASB11 in BIK regulation, we transfected Flag-ASB11 into 293T cells and then investigated its influence on BIK homeostasis. We found that the cellular abundance of BIK protein was reduced by the overexpression of ASB11 (Fig. 4). Furthermore, this ASB11-promoted BIK downregulation was blocked by treatment of cells with proteasome inhibitor MG132, indicated the participation of proteasome system in BIK downregulation (Fig. 4).

Next, we employed the cycloheximide (CHX) assay, in which cells were treated with the protein synthesis inhibitor CHX to monitor the half-life of proteins. The CHX assay demonstrated a decreased stability of BIK protein in ASB11 overexpressed 293T cells (Fig. 5). Together, our results indicate that ASB11 promotes BIK degradation through ubiquitin-proteasome pathway.

ASB11 promotes cell survival and antagonize the DNA damage-induced cell death

Based on the fact that BIK is induced by the DNA damage response to promote apoptosis, we reasoned that overexpression of ASB11 might be able to antagonize the DNA damage-induced cell death. To verify this notion, we analyzed the influence of ASB11 overexpression on the sensitivity of cancer cells to DNA damage agents. We established two HCT116 cell lines that stably expressed wild-type and SOCS box truncated ASB11 mutant, which cannot form Cul5 ubiquitin ligase complex. The cells

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were treated with DNA damage agent doxorubicin, etoposide, or cisplatin for 48 hr followed by applying PI (propidium iodide) stain and flow-cytometry to monitor the sub-G1 (apoptotic) cells. We found that ASB11 overexpression induced a pro-survival effect as it partially inhibited the DNA damage-induced apoptosis (Fig. 6). In contrast, the overexpression of SOCS box truncated ASB11 did not promote cell survival (Fig. 6), indicating that the pro-survival function of ASB11 is dependent on the formation of Cul5-ASB11 complex. Together, our results suggest that ASB11 promotes cell survival and antagonizes the DNA damage agents induced apoptosis by facilitating the degradation of pro-apoptotic BIK protein through the ubiquitin-proteasome pathway.

ASB11 is transcriptionally repressed by DNA damage in a p53-dependent manner

After demonstrating a blockage effect of ASB11 on DNA damage-induced cell death, we wanted to investigate whether ASB11 could be regulated in response to DNA damage. To this end, we treated HCT116 cells with DNA damage agent doxorubicin, 5-fluorouracil, or cisplatin for 24 hr and found that each of these agents led to a dose-dependent downregulation of ASB11 mRNA level (Fig. 7). Since p53 is a key player of the DNA damage response, we next examined the contribution of p53 pathway to DNA damage-induced ASB11 downregulation. Remarkably, in p53-deficient HCT116 cells, ASB11 mRNA levels were unchanged or even slightly elevated in response to DNA damage agents (Fig. 7). These findings indicate that DNA damage represses ASB11 mRNA through a p53-dependent mechanism.

To further evaluate the capability of p53 in regulating ASB11 mRNA, we transfected p53-null H1299 cells with p53 and found that this ectopic p53 induced a decrease of ASB11 mRNA even without DNA damage. Likewise, p53-proficient

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HCT116 cells expressed a lower level of ASB11 mRNA than that in p53-deficient HCT116 cells (Fig. 8). These findings indicate that ASB11 is transcriptionally suppressed by p53.

DNA damage prevents BIK from ASB11-mediated ubiquitin-proteasomal degradation in a p53 dependent manner

Consistent with the DNA damage-induced ASB11 downregulation and ASB11-induced BIK degradation, BIK protein level was elevated in HCT116 cells in response to DNA damage agent doxorubicin, 5-fluorouracil, or cisplatin. However, this elevation was greatly impaired in p53-deficient HCT116 cells (Fig. 9). By treating cells with proteasome inhibitor MG132, we showed that DNA damage-induced BIK elevation was mainly due to an inhibition of its proteaosmal degradation, as MG132 greatly increased BIK level in cells without receiving DNA damage agents but not in damaged cells (Fig. 10a-c, right panels). This difference in BIK proteasomal degradation seen in the damaged and undamaged conditions was greatly diminished in p53-deficient HCT116 cells (Fig. 10a-c, left panels). Furthermore, in vivo ubiquitination assay showed that DNA damage agent doxorubicin and 5-fluorouracil decreased the ubiquitination level of BIK in p53 proficient HCT116 cells but not in p53 deficient HCT116 cells (Fig. 11). Together, these data support an idea that DNA damage acts through p53 to downregulate ASB11, thereby preventing BIK ubiquitination and degradation.

To strengthen this idea, we further tested the contribution of ASB11 to DNA damage-induced BIK stabilization. CHX assay demonstrated an increased BIK half-life by cisplatin treatment. However, in ASB11 knockdown cells, such BIK stabilization is largely compromised (Fig. 12). Taken together, we provide a novel model in which the

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DNA damage-induced p53 pathway transcriptionally suppresses ASB11 to inhibit ASB11-mediated ubiquitination and proteasomal degradation of BIK protein.

ER stress transcriptionally upregulates ASB11 expression and promoted BIK degradation in the XBP1 dependent pathway

Having discovered a role of ASB11 in DNA damage-induced BIK stabilization, we wondered whether ASB11-mediated BIK ubiquitination can also be regulated under other stressed conditions. Through literature search, we found that both ASB11 and BIK are ER resident proteins [78, 125]. Furthermore, BIK is involved in calcium homeostasis function of ER [126] and is regulated by ER stress sensor GRP78 [68]. Based on these findings, we decided to explore a linkage of ER stress to ASB11-mediated BIK ubiquitination..

We used ER stress inducer thapsigargin and tunicamycin, which disturbs protein homeostasis in ER by alternating ER calcium homeostasis and blocks protein N-glycosylation, respectively, to activate the UPR. After 24 hours treatment with thapsigargin and tunicamycin, the mRNA level of ASB11 was significantly upregulated in the 293T cells (Fig. 13). Furthermore, knockdown of XBP1 blocked the induction of ASB11 expression, indicating that ER stress response promotes ASB11 expression through the IRE1-XBP1 pathway (Fig. 13). In line with this finding, the BIK protein abundance was decreased by tunicamycin treatment in 293T cells (Fig. 14). Furthermore, this BIK downregulation was abolished by MG132 treatment (Fig. 14), indicating that ER-stress response promotes BIK degradation through the ubiquitin-proteasome system. We further showed that ER-stress inducers failed to downregulate BIK protein abundance in the XBP1 knockdown 293T cells (Fig. 15). Thus, our

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findings indicate that ER stress acts through IRE1-XBP1 pathway to induce ASB11 expression, thereby promoting BIK degradation.

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IV. Discussion

In this study, we revealed a novel molecular mechanism by which the live/death decision of human cells is regulated by the ASB11-mediated ubiquitination and proteasomal degradation of BIK. In this model, ASB11 was identified as the SRS that recruits BIK to the Cul5-based ubiquitin E3 ligase complex for BIK ubiquitination. This conclusion is based on several lines of evidence. First, the interaction of ASB11 and BIK was demonstrated by the Co-IP and in vitro binding assay. Second, in vitro ubiquitination assay demonstrated the capacity of reconstituted ElonginBC-Cul5-ROC2-ASB11 E3 complex to promote polyubiquitination of BIK *in vitro*. Third, overexpression of ASB11 reduced the stability and abundance of BIK protein in a proteasome dependent manner, indicating that ASB11 promoted BIK degradation through ubiquitin-proteasome system. Together, our study identified ASB11-based Cul5 complex as an ubiquitin ligase for BIK.

After the discovery of ASB11-mediated BIK ubiquitination/proteasomal degradation pathway, we further investigated the physiological roles and biochemical regulation of this machinery. In the DNA damage-induced cell death, it is known that BIK is transcriptionally upregulated by p53 to promote apoptosis. Here, we identified an additional mechanism that facilitates BIK accumulation upon DNA damage through the blockage of ASB11-mediated proteasomal degradation of BIK protein and indicated the contribution of this BIK stabilization to DNA damage-induced apoptosis. These conclusions are based on following findings. First, ASB11 transcription is repressed in response to several DNA damage agents through a p53-dependent manner. Second, DNA damage downregulated BIK ubiquitination level in a p53-dependent manner.

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Third, DNA damage diminished BIK proteasomal degradation through a p53-dependent manner. Consistent with these observations, ASB11 overexpression inhibits DNA damage-induced apoptosis and this effect is dependent on the formation of ASB11-containing Cul5 complex. Despite these findings, the detailed mechanism by which ASB11 transcription is repressed by DNA damage/p53 pathway remains to be investigated. It is still unknown that whether ASB11 is directly targeted or indirectly regulated by p53. The analysis performed by PROMO, an online bioinformatics program specifically designed to identify putative transcription factor binding sites, indicated the existence of putative p53 binding sites on the promoter of ASB11. However, genome-wide studies did show that p53 represses genes expression mainly through the indirect but not the direct pathways[127]. In the future, the putative p53 binding sites will be examined by employing the ChIP assay to further investigate the mechanism of p53-suppressed ASB11 expression.

In addition to the DNA damage response, we found that ER stress can also regulate the expression of ASB11 and BIK. However, in contrast to the downregulation of ASB11 in DNA damage response, ASB11 mRNA is upregulated by UPR in response to the ER stress. We demonstrated that ASB11 is transcriptionally upregulated through IRE1-XBP1 pathway in 293T cells treated with ER stress inducers tunicamycin and thapsigargin. Consistent with this result, the ubiquitination and proteasomal degradation of BIK were both induced by tunicamycin treatment through IRE1-XBP1 pathway. These findings implied that ASB11 is involved in the adaptive response of UPR by mediating BIK ubiquitination and degradation. Based on the fact that BIK was reported to be binding and inhibited by the ER stress sensor GRP78 [68], it is interesting to know whether BIK could be released from GRP78 and then activated in the early stats of ER

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stress response, right after GRP78 is occupied by the unfolded proteins. If so, ASB11 may play a critical role in the pro-survival pathway in the early state of UPR. However, more studies are necessary to support our hypothesis. In the future, we will exam the influence of ASB11 on the sensitivity of cells to ER stress agents. If ASB11 indeed plays critical role in the pro-survival adaptive response of UPR, knockdown of ASB11 should sensitize cells to the ER stress agents. Furthermore, we will evaluate whether the elevated ubiquitination and proteasomal degradation of BIK is dependent on the upregulation of ASB11 in the ER stress response. Finally, the mechanism by which XBP1 regulates ASB11 will also be determined. XBP1 is a transcription factor and PROMO analysis identified a number of XBP1 binding sites on ASB11 promoter. Future studies will determine the critical region in this promoter responsible of XBP1-mediated transactivation and the direct or indirect role of XBP1 in promoting ASB11 transcription.

In this thesis, we uncover the regulatory mechanisms for ASB11-based Cul5 ubiquitin ligase in response to different stress conditions to alter the stability of a proapoptotic protein BIK. The opposite regulation of ASB11 by DNA damage and UPR would lead to opposite outcomes in the live/death decision of cells. Besides the understanding of basic molecular mechanism of cell fate decision mediated by ASB11, our study also offers important clinical implications by exploiting this function of ASB11. For instance, our finding of the pro-survival role of ASB11 in response to DNA damage agents suggests that ASB11 can be a novel target for combination therapy to sensitize cancer cells to chemotherapeutic agents. This notion is supported by a previous study demonstrating that treatment of proteasome inhibitor bortezomib induces BIK protein accumulation to sensitize otherwise resistant head and neck squamous cell

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carcinomas to cisplatin treatment [128]. In contrast, inhibition of ASB11 may be a beneficial way to overcome the adaptive response of UPR and then sensitize cancer cell to ER stress inducers. Furthermore, UPR and ER stress-induced apoptosis are also be involved in pathologic conditions other than cancer, such as neurodegenerative diseases, diabetes mellitus, and ischemia. It is interesting to know that what is, if any, the role of ASB11-mediated BIK ubiquitination in those conditions [129].

In conclusion, our study uncovers a BIK ubiquitination and degradation mechanism mediated by ElonginBC-Cul5-ROC2-ASB11 ubiquitin ligase complex. We further discover the opposite regulations of ASB11 by DNA damage and ER stress to affect the proteostasis of BIK thereby influencing on the live/death decisions of cells under these stressed conditions (Fig. 16). Based on our results, we suggest that ASB11 can be a potential target for certain diseases, such as cancer and neurodegenerative diseases. Together, this thesis provides novel and interesting insights into the DNA damage-induced and ER stress-induced apoptosis pathways and implies new therapeutic strategies for devastating human diseases.

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VI. Figures

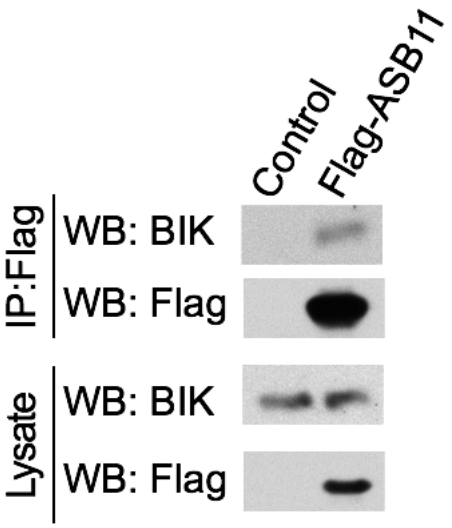

Fig.1 Immunoprecipitation assay for the interaction between ASB11 and BIK.
293T cells were transfected with control plasmid or Flag-ASB11 at two days before cell lysates preparation. Anti-Flag M2 beads were used to precipitate Flag-ASB11 from cell lysates.

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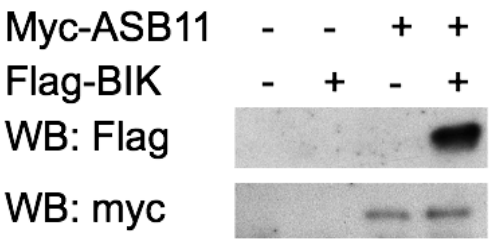

Fig.2 *In vitro* binding assay for detecting the interaction between ASB11 and BIK. Myc-ASB11 was purified by Anti-Myc beads and incubated in the RIPA buffer with or without purified 3xFlag-BIK. Then the beads were washed and bound proteins were detected by western blot.

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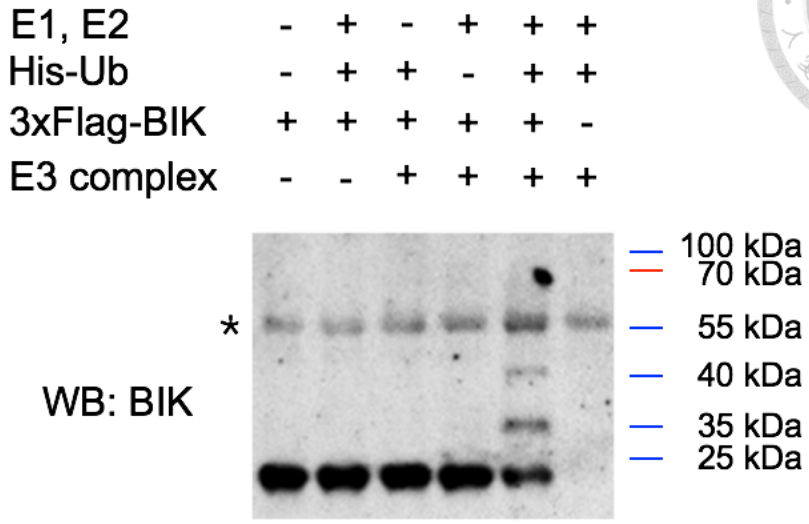

Fig.3 ElonginBC-Cul5-ROC2-ASB11 complex promotes BIK polyubiquitination *in vitro*.

Flag-ASB11, Myc-Cul5, HA-Elongin B, T7-Elongin C and V5-ROC2 were co-transfected into 293T cells and the entire E3 ligase complex was purified by Anti-Flag M2 beads.3xFlag-BIK purified from transfected cells was eluted by Flag-peptide. The E3 ligase complex was incubated with E1, E2, His-Ub and Flag-BIK in ubiquitination reaction at 37℃ for 165 mins. The Reaction products were detected by Western blot. Asterisk marks a non-specific band.

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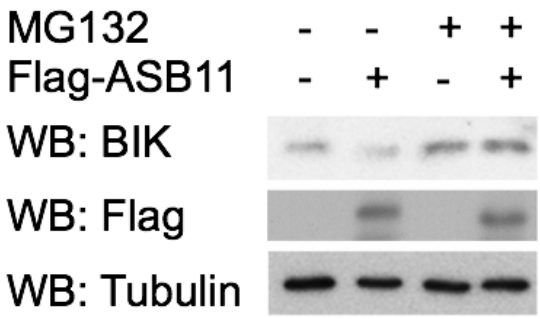

Fig.4 ASB11 promotes BIK degradation through ubiquitin-proteasome pathway.
Western blot analysis of BIK abundance in 293T cells transfected with ASB11 or vector and treated with or without MG132.

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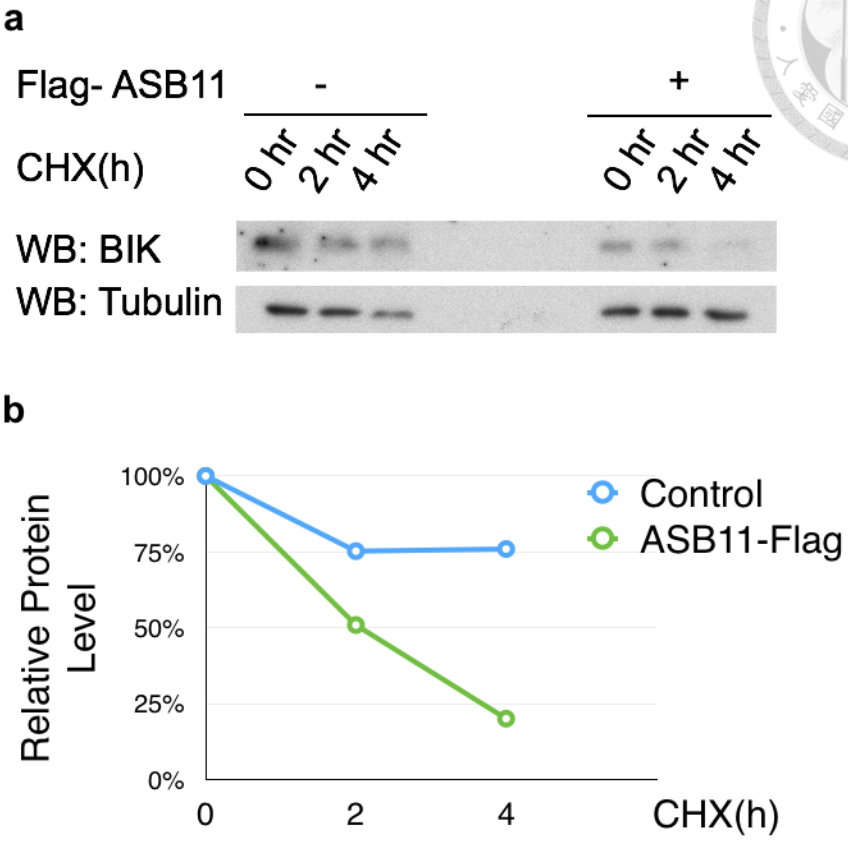

Fig.5 Ovrexpression of ASB11 reduces BIK stability.
(a)293T cells were transfected with or without Flag-ASB11 at 2 days before cycloheximide (CHX) assay. Cells were harvested at indicated time points after 100 μg/ml CHX treatment. (b) Quantitative analysis of (a).

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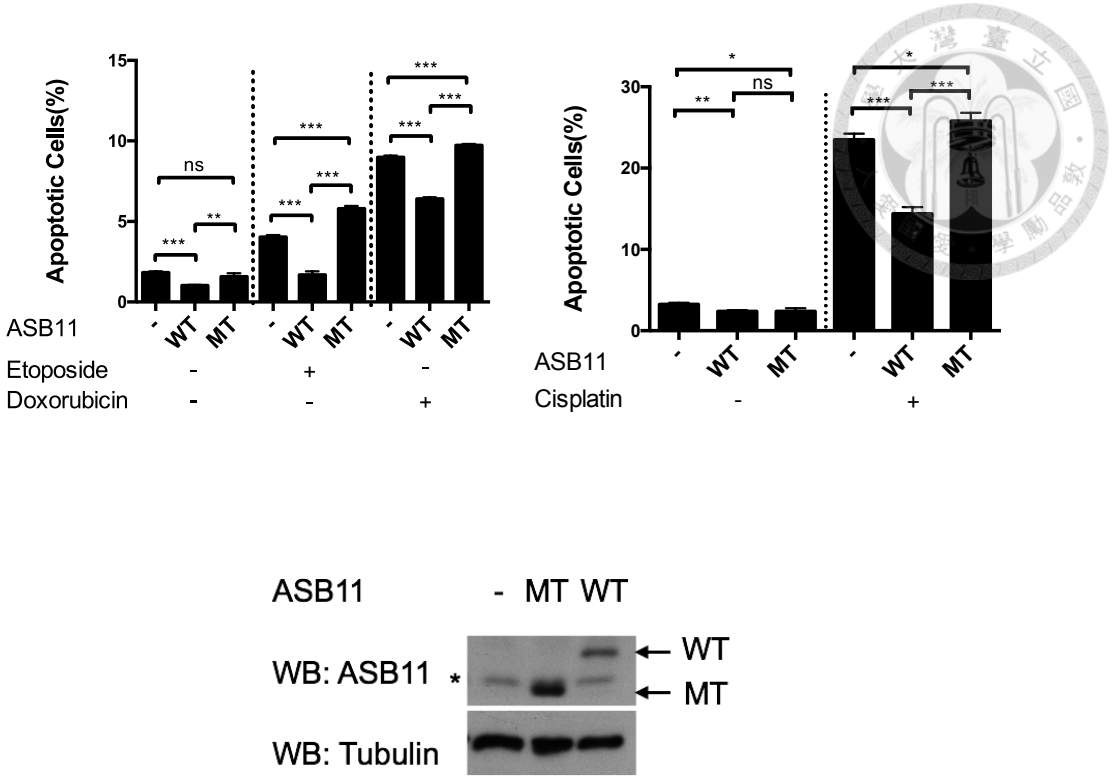

Fig.6 ASB11 impairs DNA-damage agents induced apoptosis.
HCT116 cells stably expressing wild type ASB11 or its mutant were treated with 5 μM Etoposide, 1 μg/ml doxorubicin, or 10 μM cisplatin for 24 hr. cells were harvested for PI staining, followed by flow cytometry analysis of apoptotic cells. **P*<0.05; ***P*<0.01; ****P*<0.001. The expression levels of wild type and mutant ASB11 are shown.

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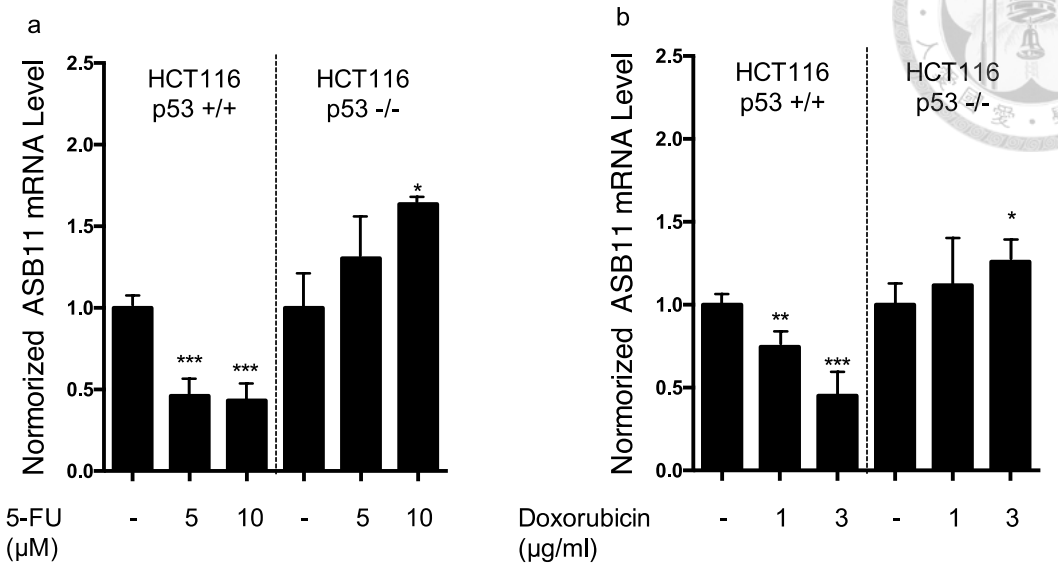

(By Fei-Yun chen)

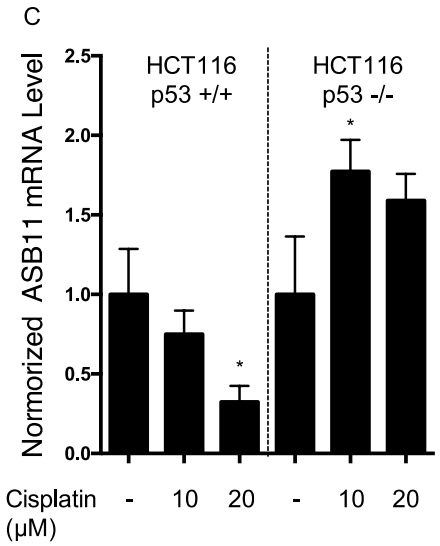

Fig.7 DNA damage agents downregulate ASB11 expression in a p53 dependent manner.

p53 proficient and deficient HCT116 cells were treated with indicated agents for 24 hr. Cells were harvested for RT-qPCR analysis of *ASB11* expression. (a)(b) Were done by Fei-Yun chen.

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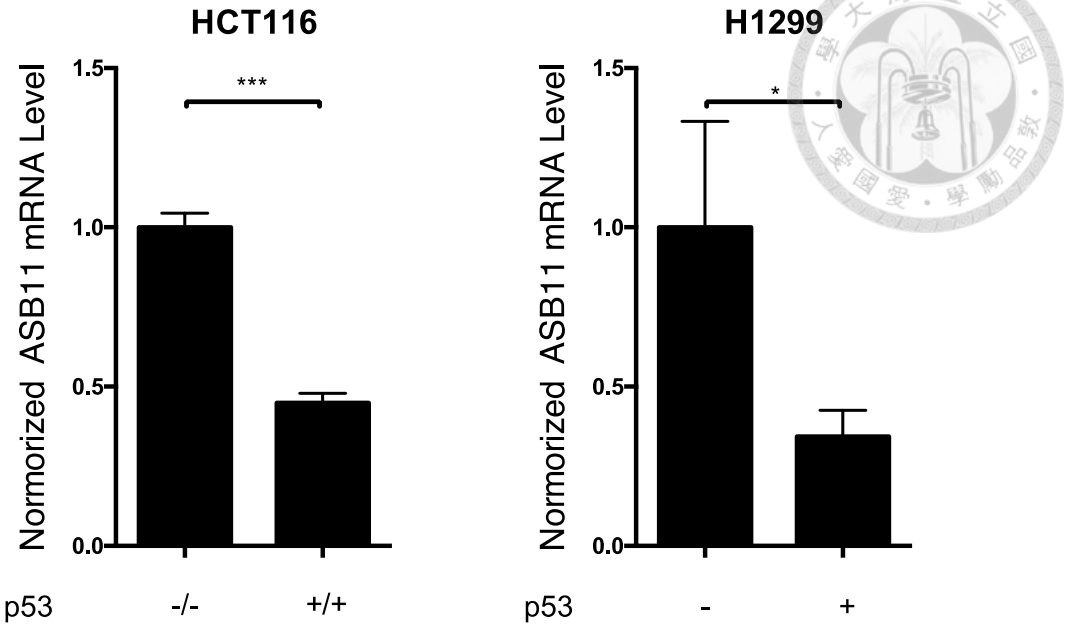

Fig.8 p53 represses *ASB11* mRNA expression.
RT-qPCR analysis of *ASB11* mRNA levels in p53-rpoficient and deficient HCT116 cells or H1299 cells stably expressing control vector or p53. **P*<0.05; ****P*<0.001.

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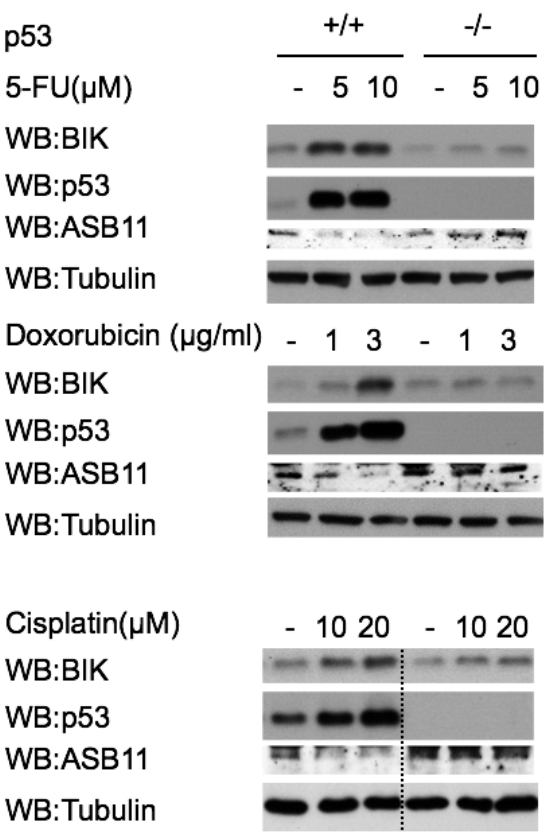

(By Fei-Yun chen)

Fig.9 DNA damage agents downregulate ASB11 protein level in a p53 dependent manner and increase BIK expression.
p53 proficient and deficient HCT116 cells were treated with indicated agents for 24 hr. Cells were harvested for Western blot analysis. (By Fei-Yun chen)

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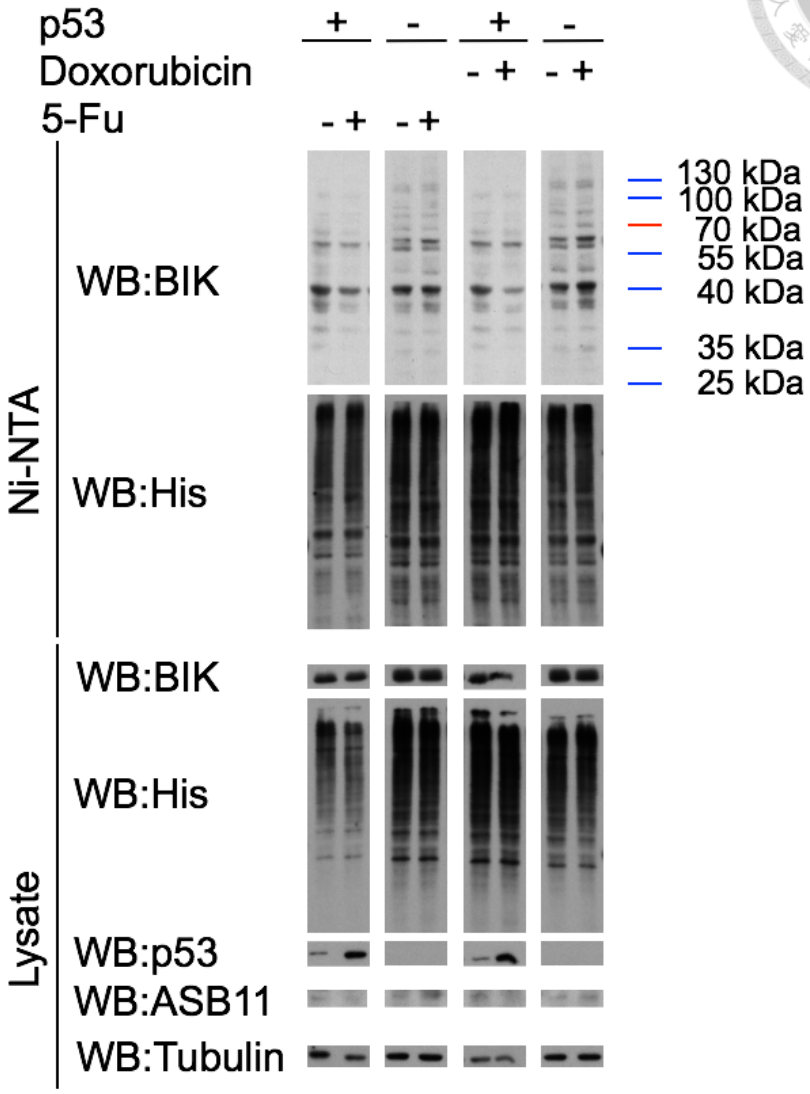

Fig.10 DNA damage agents reduce BIK polyubiquitination in a p53-dependent manner.

One day after transfection with His-Ub and BIK, 293T cells were treated with 2 μg/ml Doxorubicin or 10 μM 5-Fluorouracil (5-FU). Six hours later, 1 μM MG132 was added to transfectants and incubated for 18 hr. Ubiquitinationed proteins were precipitated from cell lysates by Ni-NTA beads and detected by Western blot.

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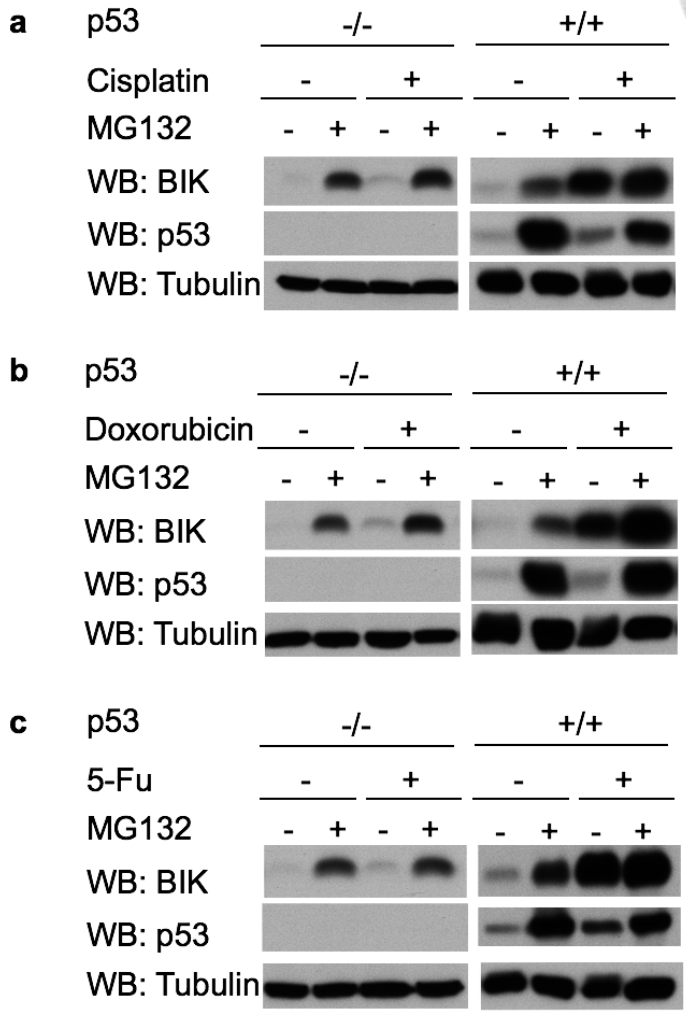

Fig.11 DNA damage agents prevents BIK from proteasomal degradation in a p53 dependent manner. p53-proficient and deficient HCT116 cells were treated with 25 μM Cisplatin (a), 2 μg/ml Doxorubicin (b) or 10 μM 5-FU (c). Six hours later, 1 μM MG132 was added to media and incubated for 18 hr. Cells were harvested for Western blot analysis

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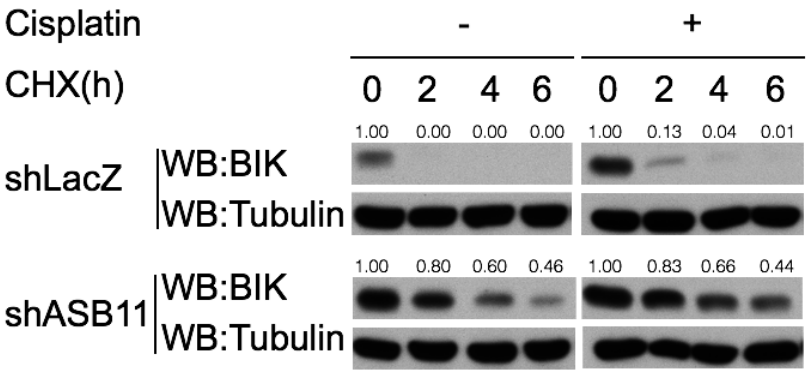

Fig.12 Cisplatin stabilizes BIK in control shRNA expressing cells but not in ASB11 knockdown cells.

p53-proficient HCT116 cells stably expressing LacZ or ASB11 shRNA were treated with 100 μg/ml cycloheximide for indicated time points before harvest for Western blot analysis.

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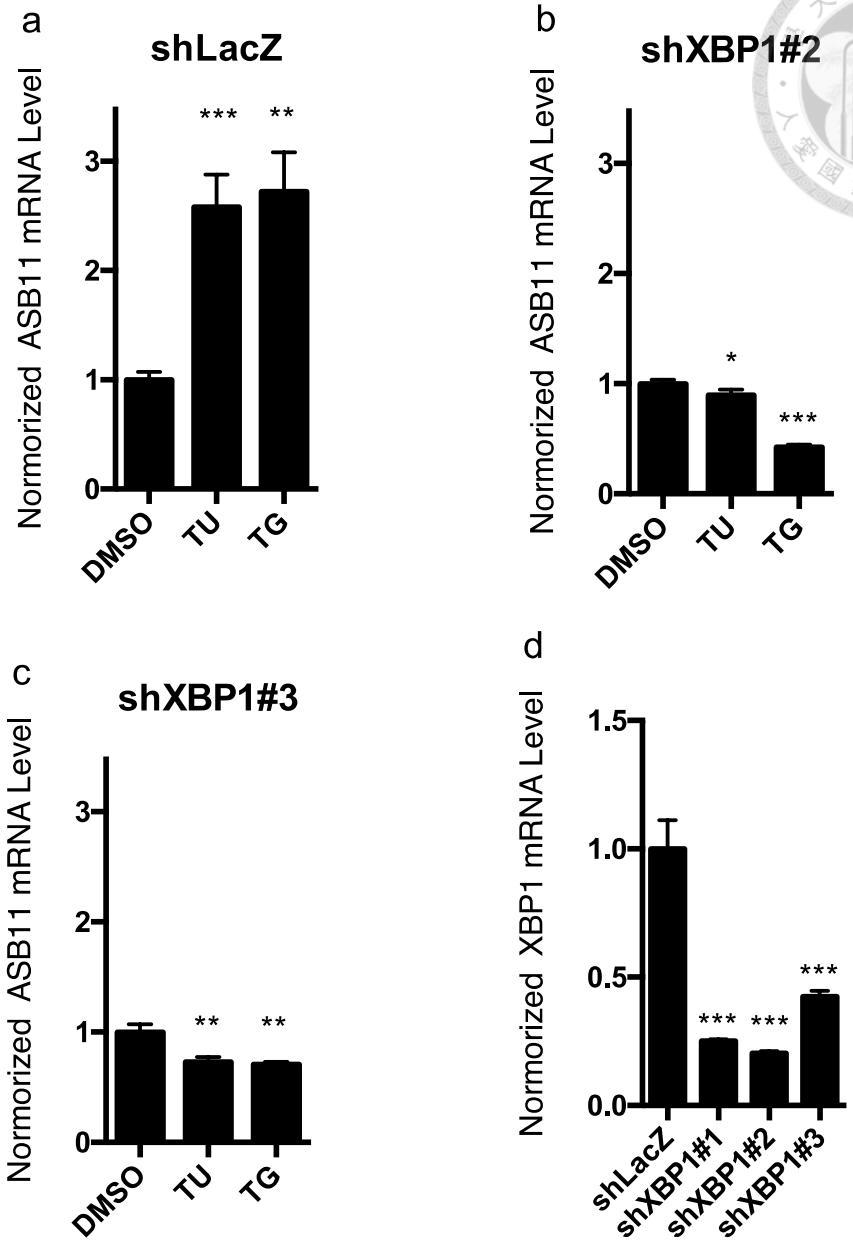

Fig.13 ER-stress inducers upregulate *ASB11* expression through XBP-1 pathway. (a-c) 293T cells stably expressing LacZ or XBP1 shRNAs were treated with 200 ng/ml tunicamycin (TU) or 80 nM thapsigargin (TG) for 24 hr and then harvested for RT-qPCR analysis. (d) Knockdown efficiencies of XBP-1 shRNAs.

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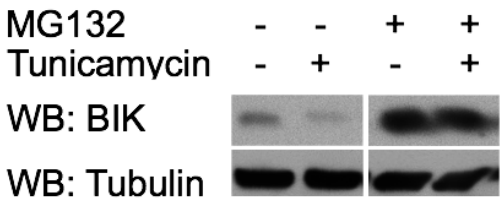

Fig.14 ER-stress promotes BIK degradation through ubiquitin-proteasome pathway.
293T cells were treated with 2 μg/ml tunicamycin. Six hours later, MG132 was added into media and incubated for 18 hr. Cells were harvested for Western blot.

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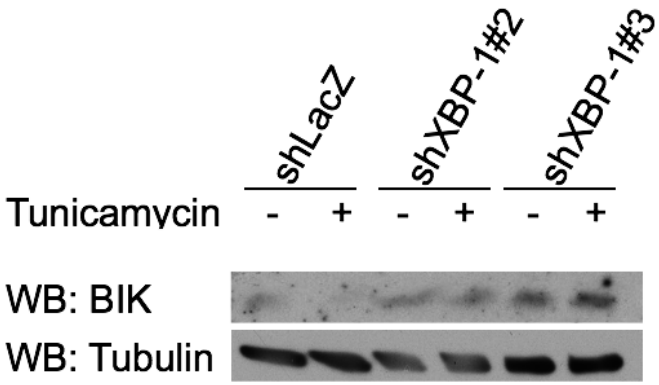

Fig.15 ER-stress promotes BIK degradation through XBP-1 dependent pathway.
293T cells stably expressing LacZ or XBP1 shRNAs as in Fig. 13 were treated with 2 μg/ml tunicamycin for 24 hr and then harvested for Western blot analysis

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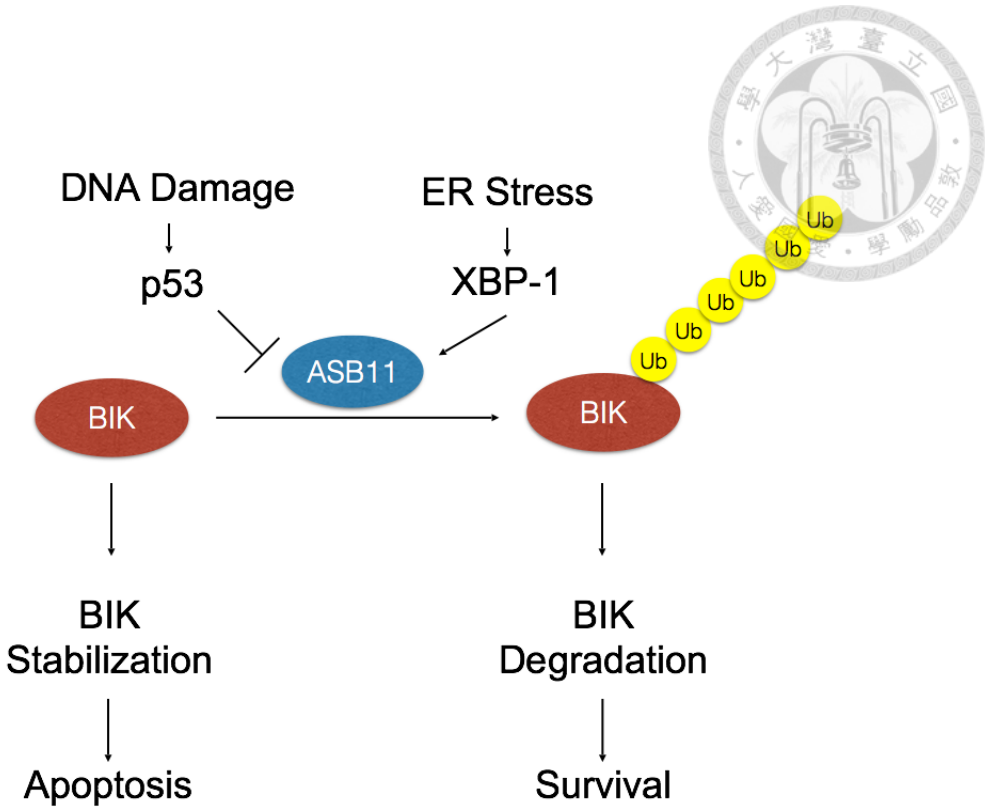

Fig.16 The working model for the role of ASB11 in BIK ubiquitination and apoptosis regulation.
In this model, BIK is ubiquitinated by ElonginBC-Cul5-ROC2-ASB11 ubiquitin ligase complex and then be degraded through the proteasomal system. The mRNA level of ASB11 is upregulated by ER stress response and downregulated by DNA damage response through XBP1- and p53-dependent pathway, respectively. Base on our results, we suggested that ASB11 plays a role in the live/death decision of the cells under stress conditions.

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