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Master Thesis

內質網壓力促進BIK泛素化機制及其治療上的應用

Identification of ER stress induced BIK ubiquitination pathway and its therapeutic application

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口試委員會審定書

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Identification of ER stress induced BIK ubiquitination pathway and its therapeutic application

本論文係黃敏瑜君（R04B46031）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 107 年 7 月 6 日承下列考試委員審查通過及口試及格，特此證明。

口試委員：_____（簽名）

（召集人）

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_____ _____

_____ _____(指導教授)

謝辭

兩年的時間就在收 Lysate、Western Blot、壓片、MTT、抽 midi、ChIP、產 Virus、養細胞、動物實驗、聊天、嬉鬧當中渡過了。

謝謝老師兩年來每次的 meeting 都提供了不同的建議和實驗方向，讓實驗都在正確的軌道上。謝謝老師在最後的一個月密集的幫我改英文和論文，每次跟老師討論都讓我佩服老師的心思縝密，老師給的意見總是彌補了我的不足，也提供了新的思考方向。謝謝飛澐學長一步一步的帶著沒有實驗室經驗的我做實驗，每每我出錯時，謝謝你總是幫我想解決辦法或者和我一起承擔。謝謝飛澐學長就像是我的第二個指導老師一樣，在 lab meeting、進度報告前，都會仔細地聽我演練、幫我修改，也謝謝學長常常配合我無理取鬧的捉弄。謝謝文新學長，教我動物實驗，每天不厭其煩的來和我說說笑笑，消除了許多做實驗的煩躁。謝謝資彧、閔棋、丁丁、欣欣、鈺軒學長、忻怡學姊、佩蓉學姊，在實驗上和生活上都幫忙很多，在繁忙之餘還是可以一起聊天、玩樂。最後也謝謝家人的支持，才能讓我在碩士期間能夠無憂無慮的在實驗室學習。

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中文摘要

BIK (Bcl2-interacting killer)屬於 Bcl2 家族中 BH3-only 的促進細胞凋亡蛋白。BIK 藉由跟抑制細胞凋亡蛋白包括 Bcl-2、Bcl-xL 和 Mcl-1 相互作用進而促使細胞走向細胞凋亡。BIKDD 是一個持續活化的 BIK 變異，能透過和抑制細胞凋亡蛋白有更好的相互作用，來更加增強細胞走向細胞凋亡的功能以達到殺死癌症細胞的目的。然而 BIK 和 BIKDD 的半衰期很短，蛋白不穩定性成為 BIK 在癌症基因治療上的限制。因此，了解 BIK 和 BIKDD 的降解機制就變得相當重要。使用 dominant-negative 形式的 Cullin 和基因表現降低技術，我們發現 Cul5 參與在 BIK 的泛素化和降解中。在所有 Cul5 的受質連接體中，我們經由泛素化分析確認 ASB11 可以增加 BIK 的泛素化，反之，不能連接 Cul5 的 ASB11 變異不行。在多株不同的細胞中降低 ASB11 的表現量會使 BIK 上升，暗示著 ASB11 所促進的泛素化會使 BIK 降解。另外，我們也在泛素化分析中發現，ASB11 可將泛素加在 BIK K115 和 K160 的位置上並使得 BIK 被降解。了解了 BIK 的泛素化降解機制後，接下來，我們想知道怎樣的生理情況可以調控 BIK 的 ASB11-based Cul5 降解機制。我們發現 ASB11 的 mRNA 和蛋白皆會在加入促進內質網壓力藥物 tunicamycin 和 thapsigargin 時會有上升的情形。結果顯示，在許多不同的細胞株中，內質網壓力都可以透過 ASB11 的上升來加速降解 BIK 使得 BIK 的半衰期縮短。另外我們進一步發現，ASB11 的上升源自於內質網壓力所導致的 UPR 之一—IRE1α/XBP1。活化的 IRE1 會促使轉錄因子 XBP1 進入細胞核，進而使下游基因的表現，而我們認為 *ASB11* 就是 XBP1 的轉錄目標基因。從染色體免疫沉澱實驗的結果發現，XBP1 的確可以結合到 *ASB11* 啟動子上。因此，我們提出一個模式，XBP1 會和基礎表現的 NF-Y 形成複合體，並且藉由結合 NF-Y，結合到 NF-Y 在 *ASB11* 啟動子上的結合位，以增加 *ASB11* 的轉錄。另外，我們也確認了 BIKDD 也和 BIK 走相同的機制，在內質網壓力的情況下，ASB11-based Cul5 降解機制會被提升使得 BIKDD 的穩定性下降。因此，我們假設抑制 IRE1α/XBP1

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可以穩定 BIKDD，並且增強殺死腫瘤細胞的效果。結果發現，使用 IRE1 抑制劑 STF-083010 結合 BIKDD，成功的在數個三陰性乳癌(TNBC)細胞株和裸鼠中都比單獨使用 BIKDD 有更好的治療效果，成功的應證了我們的假設。總結以上，本篇研究找到了 BIK 的降解機制，並且發現此機制在內質網壓力下會被提升，並且在細胞試驗和老鼠層次中我們發現，結合抑制劑和 BIKDD 可以在三陰性乳癌有加乘的治療效果。

關鍵字：BIK、泛素化、內質網壓力、抗癌療法

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Abstract

BIK (Bcl2-interacting killer) is a pro-apoptotic BH3-only protein of Bcl2 family. BIK interacts with the anti-apoptotic proteins, including Bcl-2, Bcl-xL and Mcl-1 to neutralize their function. BIKDD, a constitutively active mutant of BIK with an enhanced interaction with anti-apoptotic proteins, has been shown to elicit an anti-cancer activity. However, BIKDD is of a short half-life, which limits its tumor-killing effect. Thus, it would be important to unravel the degradation mechanism of BIK and BIKDD. Using dominant-negative mutants and knockdown approaches, we identify a role of Cul5-based ubiquitin ligase in mediating BIK degradation. Among the substrate adaptors of Cul5, ASB11, but not its mutant, promotes BIK ubiquitination. Knockdown of ASB11 in multiple cell lines elevates BIK level, indicating that ASB11-mediated ubiquitination leads to BIK degradation. We further identify the critical role of BIK K115 and K160 resides for its ubiquitination and degradation by ASB11. Next, we explore the physiological conditions that could regulate this BIK ubiquitination pathway. Importantly, ER stress inducers tunicamycin and thapsigargin upregulate ASB11 mRNA and protein. As a consequence, ER stress increases BIK protein turnover via proteasome and decreases BIK steady-state level in multiple cell lines. We further show that the IRE1α/XBP1 axis of ER stress-induced unfolded protein responses is responsible for ASB11 upregulation and that *ASB11* is a transcriptional target of XBP1.

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Our findings support a model that XBP1 can form a complex with the basal transcription factor NF-Y. This complex is recruited to the NF-Y binding site in *ASB11* promoter to evaluate *ASB11* transcription. Finally, we tried to apply our findings to the gene therapy treatment of triple negative breast cancer (TNBC). Similar to BIK, BIKDD is also subjected to ASB11-dependent ubiquitination and degradation, which are also promoted by ER stress. This raises the possibility for targeting the IRE1α/XBP1 axis to enhance the anti-tumor activity of BIKDD. Indeed, MTT assay show that combination of IRE1 inhibitor, STF-083010 with BIKDD has a better killing effect than BIKDD alone on several TNBC cell lines. Furthermore, the combined treatment strategy also has a better tumor killing effect in the mouse model. Thus, our findings identify a BIK ubiquitination pathway, uncover the promotion effect of ER stress on this pathway, and highlight the potential of targeting this pathway combined with active BIK for anti-cancer therapy.

Key words: BIK, ubiquitination, ER stress, anti-cancer therapy

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Content

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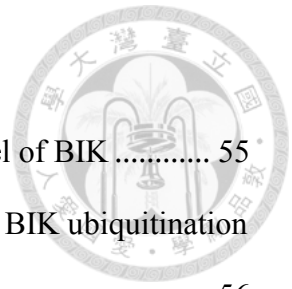

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I. Introduction

1. Apoptosis

Apoptosis, also called programmed cell death, is an important physiological process which can be used to determine cell fate and tissue homeostasis. Apoptotic cells possess the following hallmarks, chromosome condensation, DNA fragmentation, degradation of cytoskeleton and nuclear proteins, cell shrinkage, and plasma membrane blebbing (*1*). At the end of apoptosis, the apoptotic cell will be recognized and engulfed by macrophages without cell lysis and inflammatory response (*2, 3*).

Apoptosis pathway is divided into two main pathways, extrinsic and intrinsic pathways. Extrinsic pathway can initiate apoptosis by the signal of extracellular ligands such as, FasL and TNF-α. These ligands binding to death receptor, for example FasR and TNFR1, leads to the recruitment of cytoplasmic adaptor and apoptosis initiator Caspase-8, thereby forming death inducing signal complex (DISC) to initiate apoptosis by activating caspase-8 (*4*). On the other hand, intrinsic pathway initiates apoptosis by multiple intracellular stimuli (like DNA damage, ER stress or hypoxia) which results in an opening of the mitochondrial outer membrane permeabilization (MOMP) pore to release a number of apoptotic factors. The first group of apoptotic factors, including cytochrome c, Smac/DIABLO, and serine protease HtrA2/Omi, participate in the activation of caspase cascade (*5*). Cytochrome c is important for the assembly of

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apoptosome with active Apaf1 and pro-caspase-9 (*6*). Apoptosome is a quaternary protein which facilitates the dimerization and generates the catalytically active form of caspase-9 (*7*). Additionally, active Apaf1 in apoptosome can initiate the chromosome condensation (*8*). Smac/DIABLO and HtrA2/Omi can initiate apoptosis by inhibiting the function of inhibitors of apoptosis proteins (IAP) (*9-11*). The second group of apoptotic factor that released from mitochondria is involved in the late event in the commitment of cell death, which includes apoptosis-inducing factor (AIF), endonuclease G and caspase-activated DNase (CAD) (*5*). Both AIF and endonuclease G translocate to the nucleus and cause DNA fragmentation into 50~300Kb. CAD is cleaved by caspase-3 and contribute to further chromosome condensation (*8, 12*).

Extrinsic and intrinsic pathways initiate apoptosis by different mechanisms but both end at the same execution phase-the activation of executioner caspases. Caspases stand for cysteine-aspartic proteases, which specifically cleave pro-caspases at the aspartic residue and convert them to the active form. To date, major caspases have been categorized based on their function into two groups, initiators (caspase-2, -8, -9, -10) and executioners (so called as effectors) (caspase-3, -6, -7) (*13*). Executioner caspases can cleave various substrates, for example, poly [ADP-ribose] polymerase (PARP), alpha fodrin (a plasma membrane cytoskeletal protein), and NuMA (a nuclear protein). The most important executioner caspases is caspase-3 which can induce cytoskeletal

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reorganization and disintegration of the cell.

Apoptosis is important for developmental processes, ridding the body of pathogen-invaded cells and tissue remodeling in the adult (*14*). Abnormal cell death can cause a lot of diseases, including cancers (*15*). The evasion from apoptosis is sought to be one of the reasons in tumor progression (*16*). Tumor cells can be resistant to the apoptosis by the over-expression of anti-apoptotic proteins such as Bcl-2.

2. Bcl-2 family protein

The Bcl-2 (B cell lymphoma-2) family proteins are involved in the regulation of apoptotic pathway (*17*). Mutation or abnormal expression of Bcl-2 family proteins can result in cancer, neurodegenerative disorders, ischemia and autoimmune diseases (*18*). Bcl-2 family proteins share highly conserve Bcl-2 homologous domains (BH1-4) which are responsible for their function and dimerization (*19, 20*). Most of the proteins in Bcl-2 family locate on the membrane of mitochondria or ER by a hydrophobic anchor for integration into the membrane (*21, 22*). According to the structure domains and apoptotic functions, Bcl-2 family proteins are classified into three groups, the pro-apoptotic, the anti-apoptotic and the BH3 domain only proteins.

The pro-apoptotic members contain Bax, Bak and Bok. In normal cells, Bak inserts in the outer membrane of mitochondria, whereas Bax locates at cytocol (*23, 24*). During

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apoptosis, Bax will be recruited to the mitochondria and form the complex with Bak. Bax/Bak complex can form the pore like homo-oligomer, MOMP complex. The MOMP complex directly permeabilizes the mitochondria outer membrane (MOM) and results in the release of cytochrome c and apoptotic factors to turn on the mitochondria-mediate apoptosis pathway (*25*). Additionally, Bax and Bak can also localize to the ER and lead to the Ca^{2+} release to mitochondria through mitochondria-associated membrane (MAM) (*26, 27*). Ca^{2+} flux into the mitochondria can cause the cristae remodeling and the opening of permeability transition (PT) pore which also result in the release of cytochrome c (*28, 29*).

The pro-survival or anti-apoptotic members include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG and Mcl-1. Most of the proteins in this group have multiple BH domains. BH1 and BH2 contribute to the hetero-dimerization with Bax which inhibit MOMP formation and the apoptosis pathway (*30*). Furthermore, BH1, BH2, BH3 can form a "pocket", the hydrophobic groove, which is stabilized by BH4 (*31*). This hydrophobic groove can bind the BH3 domain of pro-apoptotic protein and neutralizes their function (*21, 32*).

BH3-only proteins regulate the activity of both pro-apoptotic and anti-apoptotic groups. BH3 domain only proteins are categorized into activators and sensitizers. The activators, include Bid and Bim, can bind to not only Bax and Bak which direct activate

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the formation of MOMP but also the proteins of anti-apoptotic group which neutralize their functions (*33, 34*). The sensitizers, such as Bad, Bmf, Noxa, Hrk and BIK, only bind to the anti-apoptotic proteins, thereby leaving an opportunity for the binding of activators to Bax and Bak (*25*).

Moreover, Bcl-2 family proteins can be regulated by transcription, translation, subcellular localization and post transcriptional modification which enable them to response to various extracellular stimuli and intracellular stresses (*35*). The extrinsic pathway, TNF/FAS signaling induces the cleavage of the N-terminus of Bid by caspase-8 which results in the translocation of Bid from cytosol to the outer membrane of mitochondria and dimerizes with Bcl-2 to promote the release of cytochrome c (*36*). DNA damage induces the upregulation of BIK, NOXA, PUMA and Bax by the p53 at the transcriptional level (*37-40*). ER stress responsive transcription factor CHOP can specifically bind to the promoter region of Bim, thereby upregulating Bim at the transcription level. Additionally, the dephosphorylation of Bim by phosphatase, PP2A can prevent the ubiquitin-dependent proteasome degradation under the ER stress (*41*). In addition, Bad can be phosphorylated by PI3K downstream Akt-1/PKB. Phosphorylated Bad losses the ability of forming heterodimer with anti-apoptotic Bcl-2 and cannot promote cell death (*42*).

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3. Bcl-2-interacting killer (BIK)

BIK stands for Bcl-2-interacting killer and belongs to the BH3-only Bcl-2 family. BIK can associate with anti-apoptotic proteins by its BH3 domain, including, Bcl-2, Bcl-XL and Mcl-1, and neutralizes their functions by liberating Bax (*43-45*). BIK is a 18 KDa protein and consists of 160 amino acids. BIK contains a BH3 domain which contribute to its apoptotic effect and a transmembrane domain at its C terminus which can anchor on the ER membrane (*45, 46*). The phosphorylation of serine 33 and threonine 35 residues of BIK by a casein kinase II-related enzyme promotes its apoptotic function by enhancing BIK interaction with the anti-apoptotic Bcl-2 family members (*47, 48*).

BIK induces apoptosis by releasing cytochrome c from mitochondria to activate pro-caspase-9 (*49*). BIK triggers cytochrome c releasing by blocking the formation of both Bcl-2/IP3R and Bcl-2/Bax complexes (*50*). IP3R is a receptor which responds to the Ca^{2+} release from ER, so the disassociation of Bcl-2/IP3R complex can trigger the release of Ca^{2+} from ER. On the other hand, the disassociation of Bcl-2/Bak complex increases the ER-associated Bax. ER-associated Bax can interact with DAPK1 and enhances the contact between ER and mitochondria which facilitates the Ca^{2+} flux into mitochondria through this contact (*50*). Furthermore, the Ca^{2+} release from ER results in the recruitment of Bax, Bak to mitochondria which form the MOMP complex and

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DRP1 (Dynamin-related protein 1) from cytosol to mitochondria (*51, 52*). DRP1 is a mitochondria fission protein which can remodel the mitochondrial membrane cristae to trigger apoptosis (*53*).

BIK function as a tumor suppressor and is downregulated in certain cancer types. For example, BIK is silenced by epigenetic regulation in renal cell carcinomas, whereas BIK is highly expressed in normal kidney cells (*54*). Furthermore, deletion or mutation of *BIK* gene has also been found in some cancers such as glial brain tumors, oral cancer and renal cell carcinomas (*55, 56*) Paradoxically, *BIK* mRNA is constitutively expressed or highly expressed in some breast cancer cell lines. Furthermore, BIK high expression correlates with the poor prognosis of lung and breast cancer patients (*57, 58*). However, it is found that in certain cancer cell line expressing a high level of *BIK* mRNA, BIK protein cannot be accumulated due to a rapid degradation by proteasome and this degradation is critical for the survival of cancer cells (*59*). These findings suggest that protein stability regulation may be more important than transcriptional regulation for determining BIK level in cancer cells. Another explanation for the observed high *BIK* mRNA level in certain cancer cells is that BIK-potentiated autophagic flow may benefit the progression of tumor (*60*).

It has been reported that BIK can be transcriptionally upregulated by transcription factor, E2F after treating chemotherapeutic agents and contribute to the efficient

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apoptotic response (*46, 61*). The report indicates that BIK may be a promising candidate for therapy, thereby, a new anti-tumor gene therapy has been developed based on the pro-apoptotic function of BIK (*62, 63*). This therapy used a constitutively active form of BIK, BIKDD, which replaces the Ser 33, Thr 35 residues to Asp residues for mimicking the phosphorylated form with an enhanced tumor killing effect (*64*). BIKDD successfully eradicates pancreatic, lung and breast tumors in several studies (*62, 65-67*). Moreover, the clinical trials of therapeutic gene BIKDD in pancreatic cancer have reached to phase I. However, BIKDD is of a short half-life. The treatment of proteasome inhibitor could stabilize BIKDD and enhance its tumor killing effect (*68*). Moreover, it has been reported that BIKDDA, a mutant of BIK with longer half-life can have better therapeutic effects in triple negative breast cancer (TNBC) cell line (*69*). Additionally, the treatment of DNMT1 inhibitor and histone deacetylase inhibitor also can increase the expression level BIK (*70-72*).

4. Ubiquitination-proteasome system (UPS)

The synthesis and degradation of protein are tightly regulated to maintain the protein homeostasis. UPS is a lysosomal-independent pathway which is responsible for the final journey of protein, degradation (*73*). UPS carries out its function by two steps, first ubiquitination and then recognition by proteasome (*74*). Ubiquitination is a post-

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translational modification (PTM) like a “tagging” system which can add the ubiquitin on the lysine residue of the specific substrate. Ubiquitin is a 8 KDa, 76 amino acid protein which can carry diverse signals on the substrate by mono-ubiquitination or poly-ubiquitination. Most of the K48-linked poly-ubiquitinated substrates can be recognized and then be degraded by 26S proteasome (*75*). The UPS tagging process is achieved by three enzymes, E1 (ubiquitin-activating enzymes), E2 (ubiquitin-conjugating enzymes) and E3 (ubiquitin ligase enzymes). First, E1 utilizes 1 ATP molecule to activate ubiquitin and form the thioester bond between the C-terminus of ubiquitin and a cysteine residue on E1. Second, the activated ubiquitin is then transferred to a cysteine residue of E2. Finally, E3 can specifically transfers ubiquitin from E2 to the substrate and form the iso-peptide bond (*76*). E3 serves as the key regulator in the specificity of ubiquitination, which can specifically recognize the substrate. E3 ligases are divided into two major types, really interesting new gene (RING) family and homologous to E6AP C terminus (HECT) family. The E3 of RING family transfers the ubiquitin directly to the substrate. In contrast, the HECT family catalyzes the formation of an intermediate with ubiquitin conjugated to E3 (*77*).

(1) Cullin-RING E3 ligase (CRL)

CRL is the largest E3 family in eukaryote which is composed of 8 members, Cul1,

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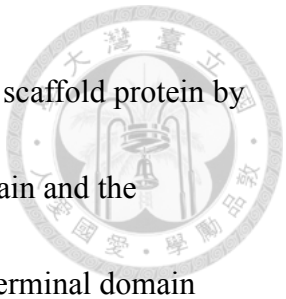

2, 3, 4A, 4B, 5, 6, 7, 9, and APC2. Cullin serves as a crescent-shaped scaffold protein by linking the substrate-recognition subunit (SRS) at its C-terminal domain and the catalytic subunit RING H2 finger protein (RBX1 or RBX2) at its N-terminal domain (*78, 79*). CRL complex can be divided into serval subtypes by their diverse SRSs, including F box, VHL, BTB, DWD, and suppressor of cytokine signaling (SOCS) proteins for CRL1, 2, 3, 4, and 5, respectively (*80*). In certain CRL complexes, there is an additional linker protein to connect SRS and Cullin, including Skp1 and DDB1 for CRL1 and CRL4, respectively. The ElonginB/C complex serves as the linker for both CRL2 and CRL5 (*81*).

CRL is regulated by neddylation, deneddylation, the turnover of SRS, and a regulatory protein called cullin-associated and neddylation-dissociated 1 (CAND1) (*81, 82*). Neddylation is an ubiquitin-like modification which can activate Cullin through a covalent binding with Nedd8. Nsddy8 triggers a conformation change which frees the catalytic domain and fills the gap between CRL and the substrate (*83*). Without neddylation, CRL complex cannot transfer ubiquitin to the specific substrate. The COP9 signalosome (CSN) is responsible for the deneddylation of Cullin, which inactivates CRL. Degradation of SRS can also be a way to regulate the function of CRL. It has been reported that binding to the overexpressed substrates can prevent the autoubiquitination and therefore stabilize SRS (*84, 85*). The stabilization of SRS can

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enhance the recruitment of specific substrate to CRL. Additionally, CAND1 regulates Cullin by binding to the both sides of un-neddylated Cullin which prevents the binding of SRS (*86*). Although initially identified as a negative regulator of CRL, recent studies indicate that CAND1 is an SRS exchange factor to accelerate the rate at which CRL equilibrates with multiple SRSs (*87*).

(2) Ankyrin repeat and SOCS box containing 11 (ASB11)

CRL5 complex contains SOCS box-containing protein, cullin5, RBX2/ROC2, Enlogin B and Enlogin C (*88*). SOCS box-containing protein family consists of a BC box which interact with EnlonginB/C and a Cul5 box which interact with Cul5. The SOCS box-containing protein family can be divided into several subfamilies depending on their additional domains, including CIS/SOCS subfamily, SPRY domain-containing SOCS box protein (SPSB/SSB) subfamily, WD repeat and SOCS box-containing protein 1 (WSB1) subfamily, Ankyrin repeat and SOCS box (ASB) subfamily, Rab40 subfamily and MUF1 subfamily (*89*). ASB11 belongs to the ASB subfamily. ASB subfamily contains 18 proteins, i.e., ASB1 to ASB18. All of them have two domains- the SOCS box in the C-terminus and the divergent N-terminal domain which is followed by different numbers of ankyrin repeats responsible for substrate recognition.

ASB11 is an ER resident protein. It has been showed that ASB11 can interact with

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ribophorin-1, an integral protein of the oligosaccharyltransferase (OST) glycosylation complex. Over-expression of ASB11 increases the ubiquitination and protein turnover rate of ribophorin-1 in vivo (*88*). The ASB11 in *Danio rerio*, d-ASB11 can regulate the embryonic as well as adult regenerative myogenesis and also the size of neuronal progenitor compartment (*90, 91*). The latter function is mediated by dASB11-induced ubiquitination and degradation of Delta, leading to the activation of Notch signaling. Thus, dASB11 is an important regulator for lateral inhibition of Delta-Notch signaling in neurogenesis (*92, 93*).

5. Endoplasmic reticulum stress (ER stress)

ER is a highly dynamic organelle in eukaryotic cells and is responsible for protein folding and secretion, calcium homeostasis and lipid synthesis (*94*). Maintenance of ER homeostasis is important because the correct folding structure, oligomerization, PTM, translocation, and glycosylation are important for the functional proteins (*95*). In the physiological conditions, many molecules in ER are responsible for protein quality control. For example, chaperon molecules-BiP (also called GRP78) and GRP94 help to stabilize the protein-folding intermediate, whereas protein disulfide isomerase (PDI) is curial for the formation of disulfide bond in oxidative environment (*96*). However, under the conditions that ER homeostasis is perturbed (called ER stress), the misfolded

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or unfolded proteins are increased, which results in the activation of unfolded protein response (UPR).

(1) Unfolding protein response (UPR)

UPR is a highly conserved pathway and consists of three branches mediated by ER stress sensors inositol-requiring enzyme-1α (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6). These three proteins are all ER transmembrane proteins. Under physiological conditions, they are inactivated due to the binding of BiP to their ER luminal domain. However, under ER stressed conditions, BiP is recruited to unfolded and misfolded proteins and is liberated from these sensors, which results in their activation.

ATF6 is localized on ER under physiological conditions by binding to BiP, which masks a COP-II binding site on ATF6 (*97*). ER stress induces BiP dissociation and reduction of the disulfide bonds on ATF6 luminal domain. These events result in ATF6 translocated to Golgi where it is cleaved by proteases S1P and S2P (*98*). This cleavage releases its N-terminal ATF6 cytosolic fragment (ATF6f), which is translocated to nucleus to serve as a transcription factor to activate the expression of many downstream genes (*99*). ATF6 activation can cause the upregulate transcription of ER-associated degradation (ERAD) and chaperon genes, and also, inhibit the lipid synthesis.

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PERK is a transmembrane protein kinase and is kept inactive by binding to BiP. BiP dissociation under ER stress triggers PERK oligomerization, trans-phosphorylation, and activation. The active PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α), which inhibits global translation to reduce the protein load of ER. However, this phosphorylation also allows the selective translation of the mRNA encoding activating transcription factor 4 (ATF4) (*100, 101*). ATF4 is translocated to nucleus where it induces the expression of genes involved in protein folding, antioxidant responses, autophagy, and amino acid synthesis (*102*).

Similar to PERK, IRE1α undergoes dimerization upon dissociation from BiP (*103*). This leads to the activation of both kinase and RNase activities of IRE1α (*104*). Active IRE1α catalyzes an unconventional splicing event to remove a 26-nt intron from factor X-box binding protein 1 (XBP1) mRNA (*105*), resulting the shift of open reading frame to generate a stable transcription factor XBP1s (*99, 106*). XBP1s controls the transcription of genes that are important in protein folding, ERAD, and phospholipid synthesis (*107*).

In addition to being activated by unfolded/misfolded proteins, the IRE1α signaling is finely tuned by a large number of proteins that are physically associated with the cytosolic domain of IRE1α. The concept of UPRosome refers to the ability of IRE1α to function as a scaffold for the assembly of a protein platform for regulating the amplitude

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of IRE1α signaling (*107, 108*). The IRE1α cytosolic domain binding partners include, Bax, Bak, Puma, Bim, BI-1, PTP1B, AIP1 and HSP72 (*109-112*). Most of these proteins enhance the signaling output of IRE1α, but Bax inhibitor-1 (BI-1) functions as a negative regulator (*113*). Interestingly, the BH3-only proteins Puma and Bim act in the attenuation phase to prolong the IRE1α signaling duration of UPR (*114*).

(2) Dynamic regulation of UPR in controlling cell fate decision

UPR initially results in several adaptive mechanisms, including the upregulation of enzymes and chaperons involving in the protein folding, the induction of ubiquitin-proteasome degradation via ERAD and lysosomal-mediate degradation via autophagy to remove damaged proteins and damaged ER, and the inhibition of mRNA translation by eIF2α phosphorylation. These effects collectively aid in protein folding and quality control, and reduction of protein influx to ER to restore ER homeostasis (*115, 116*). However, if the adaptive responses fail to revert the function and homeostasis of ER, such as in the conditions of chronic ER stress, UPR signaling becomes persistent and finally triggers cell death.

The cell death fate of UPR is also mediated by the same set of ER stress sensors. In response to the chronic ER stress, these signaling pathways are altered to induce cell death. For instance, the hyper-activation of PERK can upregulate the transcription

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factor CHOP through ATF4 (*117*). CHOP acts alone or together with ATF4 to upregulate a number of BH3-only proteins including Bim, Puma and Noxa, and downregulate Bcl-2, which collectively induce mitochondrial-dependent apoptosis (*112, 118*). In the case of IRE1α, its hyper-activation leads to the assembly into higher-order oligomer, which alters its RNase substrate specificity to degrade a large set of ER localized mRNAs, rRNAs and miRNAs and this process is called IRE1-dependent decay (RIDD) (*119-121*). RIDD results in the degradation of key folding mediators and therefore further aggravates ER stress at the latter stage. Furthermore, the degradation of miRNA regulating thioredoxin-interacting protein (TXNIP) by RIDD can activate NLRP3 inflammasome to trigger caspase-1-dependent pyroptotic cell death (*122*).

Another mechanism for determining the cell death decision upon chronic ER stress is the regulation of individual UPR signaling dynamics and kinetics. ATF6 and IRE1α pathways are attenuated in the persist ER stress. By contrast, PERK pathway is kept active until the cell death (*123*). It has been reported that IRE1α activity can enhance the cell viability and proliferation in the beginning of the ER stress which functions as an adaptive response and let the cell regenerate homeostasis (*124*). However, PERK pathway impairs cell proliferation and triggers cell death (*125*). Prolong ER stress results in the turning off of the pro-survival effect of IRE1α signaling and also enhancing the PERK-ATF4-CHOP pathway (*107*).

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6. Triple negative breast cancer (TNBC)

Patients belongs to TNBC is about 15% of all breast cancers. Five year overall survival of TNBC is about 78.6%, whereas 93% survival rate in other types of breast cancer (*126*). The definition of TNBC is referred to the cancer that do not express estrogen receptor (ER), progesterone receptor (PR), or HER-2 genes (*127*). TNBC is the most highly aggressive malignancy subtype of breast cancer. Current treatments of breast cancer rely mainly on the targeting of three markers, such as endocrine therapy and trastuzamab. However, these cannot be used in TNBC patients (*128*). To date, the main medical treatment of TNBC is chemotherapy, for example, anthracycline, taxanes, ixabepilone, gemcitabine, and platinum agent (carboplatin and cisplatin) (*129*). Due to this situation, scientists try to find out some new strategies for TNBC therapy. Most TNBCs highly express epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) which are important for the tumor survival, growth and metastasis (*130*). EGFR inhibitors and Bevacizumab which blocks angiogenesis are in phase I or II clinical trials (*129*). Furthermore, it has been reported that after receiving chemotherapy, several pathways are altered to cause resistance. These pathways may be used for targeted therapy, and therefore PARP inhibitor, PI3K inhibitors, MEK inhibitors, HSP90 inhibitors and HDAC inhibitors are under clinical investigation (*131*).

In addition to managing the chemotherapy resistance, identification of new

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markers in TNBC for targeted therapy is also underway. For example, hyper-activated cyclin-dependent kinase (CDK4) has been found in TNBCs which acts as a cancer stem cell regulator and novel prognostic marker. Inhibition of the kinase activity or the expression of CDK4 can eliminate the resistant cancer stem cells (*132*). Other potential markers, such as, CC3, ALDH1, Ki-67, and H2AX which are detected by immunohistochemistry are significantly higher in TNBC tissues than others (*133*). Nevertheless, HIF1α hypoxia pathway and the ER stress downstream XBP1 are highly active in TNBC, thereby, promote the tumorigenesis. Moreover, XBP1 can interact with HIF1α and form the transcriptional complex which regulates the expression of HIF1α targets by recruiting RNA polymerase (*134*).

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II. Materials and Methods

Cell culture

293T, 293FT, H1299 and TNBC cell lines (MDA-MB157, MDA-MB468 and Hs578T) were cultured in Dulbecco's modified Eagle Medium (DMEM). DMEM contain 1% Penicillin and Streptomycin (PS) and 10% fetal calf serum (FCS). All cells were maintained in 37℃ humidified incubator with 5% CO_2.

Transient transfection and plasmid

293T and 293FT were transfected by calcium-phosphate method, whereas Hs578T, MDA-MB157, and MDA-MB468 were transfected by TransIT-X2® Dynamic Delivery System (Mirus Bio) or Lipofectamin3000 (Invitrogen).

Plasmids that encoded His-ubiquitin, BIK wild type, BIK(K115R), BIK(K160R) , or BIK (K115R K160R) double mutants were generated from our laboratory previously. ASB11 wild type or ASB11 mutant was cloned into pRK5-Myc vector in our laboratory previously. pUK21.Claudin.VISA.BIKDD was a kind gift from Mien-Chie Hung Ph.D. laboratory.

Cell lysate preparation and western blot

Cells were lysed by 1X RIPA buffer (0.15 M NaCl, 50 mM Tris-HCl [pH 8.0],

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0.1% SDS, 1% sodium deoxycholate and 1% NP40) which contained protease inhibitors (10 mg/ml Aprotinin, 1 mM PMSF and 10 mg/ml Leupeptin) for 5 mins. The cell lysates were sonicated and centrifuged, followed by quantitation of protein level in supernatants by bradford reagent (Bio-Rad, Hercules, CA). Afterwards, cell lysates were mixed with sample buffer (10% glycerol, 50 mM Tris-HCl pH 6.8, 2% SDS, 0.01% bromophenol blue and 8% β-mercaptoethanol) and incubated at 100℃ for 5 mins to become protein samples. Protein samples were resolved by glycin or tricine SDS-PAGE and transferred onto PVDF membranes (Millipore) by transfer buffer with 10% methanol. The membranes were blocked by 3~5% skin milk for 1 h and were cut basing on the proper molecular weight of the protein. Next, membranes were soaked in indicated primary antibody (Table 1.) which diluted in 3~5% skin milk and rotated at 4 ℃ overnight. The membranes were washed by TBST (Tris buffered Saline with 0.1% Tween-20) 3 times for 5 mins each rotation in room temperature. After wash, membranes were followed by incubating in HRP-conjugated secondary antibody diluted by 3~5% skin milk for 1 h in the room temperature. At last, the membranes were washed by TBST 3 times again for 20 mins each rotation in room temperature, and the Enhanced Chemilumescent (ECL from Amersham) substrates or Immobilon Crescendo Western HRP substrate (Amersham) were added to the membrane. Finally, signals on the membranes were detected by hyperfilm in the dark room.

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Table. 1 Primary antibodies list.

Name	Dilution	Catalogue; Company
anti-BIK	1:1000	sc-1710; Santa Cruz
ASB11	1:1000	LTK BioLaboratories
anti-cleavage-PARP	1:1000	5625; Cell Signaling
anti-Cullin2	1:1000	GTX88108; GeneTex
anti-Cullin5	1:1000	GTX111041; GeneTex
anti-α-tubulin	1:10000	GTX628802; GeneTex
anti-GAPDH	1:10000	GTX627408; GeneTex
anti-6XHis tag	1:10000	631212; TaKaRa
anti-Myc tag	1:10000	GTX29106; GeneTex

In vivo ubiquitination assay

His-UB and Myc-ASB11 plasmid were transfected into 293T with wild type BIK, BIK(K115R), BIK(K160R) or BIK2KR individually. After 16 h transfection, 293T cells were treated with 1 μM/ml proteasome inhibitor MG132 (Calbiochem) overnight. Next day, 293T cells were harvested 48 h later after transfection and then lysed by lysis buffer A (6 M guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0, and 10 mM imidazole). The His-Ub in the cell lysates were pulled down by Nickel-beads (GE Healthcare Life Sciences) in lysis buffer A for 1.5 h rotation at 4℃. The pull-down lysates were washed once with lysis buffer A and three times with TI buffer (25 mM Tris-HCl, pH 6.8, and 20 mM imidazole). At last, the lysates were analyzed by western blot with primary antibody as indicated.

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Lentivirus production and infection

To generate lentivirus, 293FT was transfected with packing plasmid (pCMVΔ8.91), VSV-G plasmid (pMD.G), and indicated shRNA clone in the ratio of 7:7:1 (total 30 μg) by calcium-phosphate method. Medium were refreshed 16 h after transfection. After 48 h, medium that contained virus were collected and filtered by 0.45 μm pore-size filter. Medium with virus was centrifuged for 2 h and condensed before infection.

During infection, cells were cultured with virus medium which contained 8 μg/ml polybrene for 1 day. Medium were refreshed and the cell took a rest for 1 day. Finally, cells were selected by appropriated antibiotic.

Table 2. Lentivirus clones list.

shRNA Name	Target sequence (5'→3')	Clone ID
Cul2 shRNA #1	GCAAGCTACATCGGATGTATA	TRCN0000006525
Cul2 shRNA #2	CGTTTGCAGTTGATGTGTCTT	TRCN0000006522
Cul5 shRNA #1	GCAGACTGAATTAGTAGAAAT	TRCN0000006538
Cul5 shRNA #2	CGCTGTATTGTTTGCATGGAA	TRCN0000006540
ASB11 shRNA #1	CAGTGCTGCATGTGTCAATGT	TRCN0000164096
ASB11 shRNA #2	GGATAGCAGAAGAGATCTATG	TRCN0000425834

RNA extraction, reverse transcription and qPCR

Total RNA were extracted by TRIZOL reagent (Invitrogen). Total RNA were quantified by NanoDrop2000 and equal amounts of RNA were reverse transcribed to

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cDNA by iScriptTM cDNA Synthesis Kit (Bio-Rad). cDNA of total RNA was quantified by quantitative real-time PCR (qPCR). qPCR was performed using the Power SYBR Green PCR Master kit (Applied Biosystems) and analyzing by LC480 system. The condition for all PCR is : pre-incubation for 10 mins at 95℃ followed by amplification for 50 cycles (10 secs at 95℃, 10 secs at 60℃ and 30 secs at 72℃), melting curve analysis and cooling at last. The expression level formula of target gene is : $2^{-\Delta C_t}$ and $\Delta C_t = C_t(\text{target gene}) - C_t(\text{control gene})$. We used a house keeping gene *GAPDH* as a control gene and the sequences of PCR primers were shown in Table 3.

Table 3. qPCR primers list.

Name	Forward (5'→3')	Reverse (5'→3')
GAPDH	TGTTGCCATCAATGACCCCTT	CTCCACGACGTACTCAGCG
ASB11	CCTGCTAACCGACTATGGAGC	TAGGAGGAATCGTTCGAGTGG
NF-YB	ATGACAATGGATGGTGACAGTTC	CTAGCCACGTTTGCTATTGGA
NF-YC	GGAGGATTTGGTGGTACTAGCA	GCACTCGGAAGTCTTTCACTG

Cell viability assay (MTT)

The cells were counted and planted in the 96-well. After 16 h, the cells were transfected with BIKDD and treated with STF (Sigma) for 10 or 100 μg/ml for 48 h. MTT reagent (Sigma) working at 0.5mg/ml was added into each well and incubated in the 37℃ humidified incubator with 5% CO_2 for 1~2 h. The medium was sucked away

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carefully and 100 μl DMSO was added to each well. The absorbance at 570 nm was detected by Absorbance SpectraMax® Detection.

Chromatin immunoprecipitation assay (ChIP)

293T cells were treated with 10 μg/ml Tunicamycin (Cayman Chemical) for 4 h and then harvested in 5 ml PBS. Cells were fixed by 135 μl 37% formaldehyde and then stopped the fixation by 250 μl 0.25M glycine. Afterward, cells were lysed by ChIP lysis buffer (15mM HEPES PH 7.5, 14mM NaCl, 1mM EDTA PH 8.0, 0.5mM EGTA PH 8.0, 1% tritonX100, 0.1% Na-deoxycholate, 0.1% SDS, 0.5% N-Lauroylsarcosine) with protease inhibitors and sonicated on ice 10 times at each cycles by sonicator for 5 cycles. Lysates were rest on ice for 2 mins between each sonication cycle. After sonication, cells were centrifuged and the supernatants were quantitated by Bradford reagent. Each sample contained 0.7 mg protein and pre-clear by incubating with protein A magnetic beads (Millipore) for 1 h rotation at 4℃ to prevent non-specific bindings. ChIP level antibody (Table 4.) at 4 ℃ overnight on rotator. Next day, each sample was added 50μl protein A magnetic beads for 1 h rotation at 4℃. Beads were wash 5 times by RIPA buffer (50mM HEPES PH 7.5, 1mM EDTA PH 8.0, 1% NP40, 0.5M LiCL, 0.7% Na-deoxycholate) with protease inhibitors and 1 time by wash buffer (1XTE buffer with 50mM NaCl). After wash, proteins were eluted from magnetic beads by

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fresh elution buffer (1XTE buffer with 0.25M NaCl and 0.5% SDS) and DNA was extracted by Blood & Cell Culture DNA Mini Kit (QIAGEN). The amount of DNA was quantified by LC480 system with different primers which target on different promoter regions of XBP1.

Table 4. ChIP primers list.

Region			Sequence (5'→3')
A (-63 ~ +165)	-63 ~ -42	Forward	TCTCGGTAACAGGAGCACTGAG
	+141 ~ +165	Reverse	GATCTCTTCTGCTATCCTAGCCGC
B (-304 ~ -86)	-304 ~ -284	Forward	CTGTGGAAGCCAAGAAGTTTC
	-106 ~ -86	Reverse	TGATTTGGCGACTGCATGTGC
C (-870 ~ -664)	-870 ~ -851	Forward	CTCCAGCCTGGGCAACAGAG
	-683 ~ -664	Reverse	TTGCTTGAACCTGGGAGGGG
D (-1623 ~ -1474)	-1623 ~ -1600	Forward	CCTGCATGCTAGGAGAAATTCCCA
	-1649 ~ -1474	Reverse	AGGTCTCTGCCTTCATTCTTGGC

Table 5. ChIP primary antibodies list

Name	Catalogue; Company
anti-XBP1	619502; Bio Legend
anti-rabbit IgG	ab171870; Abcam

Animal experiment

In order to eliminate mycoplasmas contamination, Hs578T (TNBC cell line) cells were treated with BM-Cyclin (Sigma-Aldrich) before transfection and injection for two weeks. Hs578T cells were counted by the cell counter and $7*10^5$ cells were transfected with CMV-BIKDD or RK5M (control vector) by TransIT-X2 (Mirus Bio) for 16 h. After

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transfection, Hs578T rested for 1 day with fresh medium before injection. One day later, Hs578T cells were counted and $2*10^6$ Hs578T cells were re-suspended by 75 μl PBS. Equal volume of thawed matrigel (Corning) was mixed carefully on ice. The mixture was aspirated by insulin syringe and carefully injected into fat pad of five-week-old female BALB/cAnN.Cg-Foxnlnu/CrlNarl nude mice (National Laboratory Animal Center, Taipei, Taiwan). IRE1 inhibitor, STF-083010 (40 mg/kg) was treated every three days one week after tumor injection. Each tumor volume was measured every 3 days and calculated using the equation: $mm^3 = \pi/6 \times length(mm) \times (width(mm))^2$.

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III. Results

Knockdown of Cullin5 but not Cullin2 increases the expression level of BIK

Cullin family is the largest E3 family in eukaryote (*78*). Previous study in our laboratory showed that overexpression of dominant negative forms of Cullin2 and Cullin5 both resulted in the accumulation of BIK (Appendix 1). Since the dominant negative mutants of Cullin2 and Cullin5 are promiscuous due to the competition of common subunits ElonginB/C, we aimed to investigate which Cullin regulates BIK protein level. For this purpose, Cullin2 or Cullin5 was knocked down in 293T cells, which get rid of the competition of ElonginB/C under the overexpression of dominant negative mutants. The result showed that Cullin5 knockdown efficiently elevated the protein level of BIK (Figure 1A). However, Cullin2 knockdown didn't affect the protein level of BIK (Figure 1B). Thus, our data indicates that the Cullin5 ubiquitin ligase is capable of regulating BIK.

ASB11 but not its mutant promotes the ubiquitination of BIK

Next, we would like to identify Cullin5 substrate adaptor responsible for BIK regulation. The substrate adaptors of Cullin5 share a common feature with the presence of a SOCS box (Cullin5 box and BC box) (*89*). To find out the substrate adaptor specifically recognizing BIK, a previous study in our laboratory using an unbiased

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screen for the ability of known or predicted adaptors to bind BIK and finally identified ASB11 as a potential candidate. In order to confirm that ASB11 is the *bona fide* substrate adaptor for BIK ubiquitination, we performed the *in vivo* ubiquitination assay. To this end, 293T cells were co-transfected with BIK, His-Ubiquitin, and Myc-ASB11 wild type or mutant. Ubiquitinated proteins were pulled down by Ni-NTA beads under denaturing conditions and followed by western blot analysis. Our result indicated that ASB11 significantly promoted BIK ubiquitination, whereas the SOCS deletion mutant of ASB11, which cannot be assembled into Cullin5 complex, did not show this effect (Figure 2). This result supports that the ASB11-based Cullin5 ubiquitin ligase complex can stimulate BIK ubiquitination.

Knockdown of ASB11 increases the protein level of BIK

If ASB11 is the E3 ligase for BIK, it may affect the protein stability of BIK through UPS. Indeed, cycloheximide chase experiment previously performed in our laboratory showed that BIK protein turnover became slower in 293T cells carrying ASB11 shRNAs, compared with control cells (Appendix 2). We further established ASB11 knockdown lines in H1299 cells and MDA-MB157 cells. Remarkably, ASB11 knocked down in both cell types enhanced BIK protein abundance (Figure 3A, B) These results illustrate that ASB11 mediates the degradation of BIK.

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ASB11 promotes BIK ubiquitination at lysine115 and 160

Ubiquitinaton is a modification which specifically occurs on the lysine residue. Next, we intended to identify the site of BIK ubiquitination promoted by ASB11. BIK has only two lysine residues, so we generated individual and combined K-to-R mutants of BIK, including BIK 115 K-to-R mutant, BIK 160 K-to-R mutant and BIK 115 and 160 K-to-R double mutants. Ubiquitination assay was performed by using transfected 293T cells. The ubiquitinated proteins were pulled down by Ni-NTA beads under denaturing conditions and followed by western blot analysis. ASB11 promoted the ubiquitination of BIK wild type, but not the BIK 115 and 160 double mutants (Figure 4). Additionally, the BIK 115 or 160 single mutant showed decreased ubiquitination level compared to the BIK wild type (Figure 4). This result indicates that ASB11 promotes the ubiquitination at both 115 and 160 residues. Furthermore, the decreased level of ubiquitinated BIK was more significant in the Lys 160 mutant than the 115 mutant (Figure 4), indicating Lys 160 as the major ubiquitination site for the ASB11-promoted ubiquitination.

ASB11 cannot promote the degradation of BIK(2KR) mutant

Since ASB11 fails to promote ubiquitination of BIK K115R/K160R double mutants, referred to as BIK(2KR), we would like to test whether ASB11 affects the

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protein level of BIK(2KR). 293T cells were co-transfected with BIK(2KR), with or without Myc-ASB11 and followed by western blot analysis. Our data showed that the expression of Myc-ASB11 didn't affect the protein level of BIK(2KR) (Figure 5). This data indicates that BIK(2KR) will not be degraded through ASB11 by UPS and also further demonstrates that the degradation of BIK is depend on the ubiquitination.

ER stress upregulates the mRNA and protein level of ASB11 and destabilizes BIK

Having identified ASB11-based Cullin5 complex to promote the ubiquitination and degradation of BIK, we next asked under which physical condition could this BIK ubiquitination be regulated. The previous study in our laboratory found that *ASB11* mRNA was upregulated by treating 293T cells with tunicamycin (Tu) and thapsigargin (Tg), two inducers of ER stress (Appendix 3). Consistent with these findings, these two ER stress inducers also upregulated the ASB11 protein level, which was accompanied with the downregulation of BIK protein in 293T cell line (Figure 6). Tumor often exposed to the unbalanced protein homeostasis which results in ER stress. Due to this reason, we tried to see whether ER stress also downregulates BIK by regulating expression level of ASB11 in TNBC cell lines. TNBC cell lines includes Hs578T, MDA-MB157 and MDA-MB468 cells were treated with ER stress inducers (Tu or Tg) for 8 h. The total RNA was extracted from the cells and then generated the cDNA.

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cDNA was analyzed by qPCR to check the expression of *ASB11* mRNA. Remarkably, we found that both ER stress inducers resulted in 2~4 fold upregulation of *ASB11* mRNA in two TNBC cell lines (Figure 7A-B). We also analyzed ASB11 and BIK protein levels by western blot. The protein level of ASB11 was also upregulated in the present of ER stress inducers compared to the DMSO control (Figure 7C-E). Furthermore, consistent with 293T cell line, the upregulation of ASB11 was also accompanied with the decrease in BIK protein in TNBC cell lines (Figure 7C-E). Thus, our data indicate that ER stress upregulates ASB11 and downregulate BIK.

ER stress promotes BIK proteasomal degradation through an ASB11-dependent manner

Next we investigated whether the decrease of BIK protein level under ER stress is mediated by ASB11 upregulation. Thus, we knocked down ASB11 to see whether the knockdown blocks the ER stress induced BIK destabilization. ASB11 was knocked down in H1299 and MDA-MB157 by lentivirus carrying ASB11 shRNAs. After treating the ER stress inducer, tunicamycin, for 8 h, protein level of BIK was analyzed by Western blot. We found that knockdown of ASB11 abolished ER stress-induced BIK downregulation (Figure 8). Furthermore, we checked whether the downregulation of BIK under ER stress is due to ASB11-induced proteasomal degradation. To do this, we

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added the proteasome inhibitor MG132 together with ER stress inducer, tunicamycin, for 8 h. Our results showed that proteasome inhibitor rescued the downregulation of BIK in both TNBC cell lines and this effect was attenuated in cells expressing ASB11 shRNAs (Figure 9). Together, these data indicate that ER stress potentiates BIK proteasomal degradation through an ASB11-dependent mechanism.

XBP1 is recruited to the *ASB11* promoter

ER stress induces three branches of UPR pathways, including ATF6, IRE1α and PERK. Previous study in our laboratory showed that knockdown of IRE1 pathway downstream effector XBP1 blocked ER stress-induced upregulation of *ASB11* mRNA (Appendix 4). XBP1 is a transcription factor to turn on many UPR downstream gene (*112*). We therefore investigated whether ASB11 is a transcriptional target of XBP1. We performed the ChIP assay to check whether XBP1 can be recruited to *ASB11* promoter. To activate XBP1, 293T cells were treated with ER stress inducer (Tu) and then cells were harvested for chromatin immunoprecipitation (ChIP) assay with anti-XBP1 or a control anti-IgG antibody. Among the 4 pairs of ChIP primers, enhancement of promoter binding was found only by primer pair B (Figure 10A, B), indicating that XBP1 can be recruited to region B of the *ASB11* promoter.

Surprisingly, we could not find canonical XBP1 binding sequence in the *ASB11*

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promoter region covered by primer pair B (i.e., -86 to -304). Instead, we found GGTTAG sequence in this region which is the binding site for transcription factor NF-Y. It has been reported that NF-Y and XBP1 can form a dimer to be recruited to ERSE (ER stress response element), which contains both XBP1 binding and NF-Y binding sequences in previous research (*135, 136*). With this knowledge, we hypothesized that XBP1 may be recruited to the NF-Y binding site in the *ASB11* promoter by forming a complex with NF-Y. To confirmed it, we knocked down NF-YB subunit or NF-YC subunit in 293T cells (Figure 10C). Remarkably, we found that knockdown of NF-YB or NF-YC blocked the binding of XBP1 to the region B of *ASB11* promoter (Figure 10D). These results indicate that the basal transcription factor NF-Y is critical for recruiting XBP1 to the *ASB11* promoter region B.

ER stress and ASB11 promote BIKDD downregulation

BIKDD is a promising gene therapy strategy which can even eliminate cancer stem cells, but the short protein half-life of BIKDD protein is a potential limitation (*65, 68*). We would like to know whether BIKDD shares the same degradation mechanism as BIK. To this end, we co-transfected BIKDD and Myc-ASB11 into 293T cells. As our expectation, BIKDD protein level was decreased significantly in the present of Myc-ASB11 (Figure 11). Additionally, the expression of BIKDD was downregulated under

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the ER stress conditions (Figure 12). These results indicate that BIKDD can also be downregulate by ASB11 overexpression or by ER stress. Thus, blockage of ASB11-dependent pathway may extend the half-life of BIKDD.

Combined treatment of IRE1 inhibitor-STF and BIKDD enhance the tumor killing effect

We hypothesized that inhibition of the ASB11-dependent BIK degradation pathway should stabilize BIKDD to enhance its tumor killing effect. We choice STF-083010 as an IRE1 inhibitor. STF inhibits the RNA splicing of XBP1 by directly blocking the RNase activity of IRE1 without affecting the phosphorylation of IRE1 (*137*). We overexpressed BIKDD in three TNBC cell lines and treated them with STF. After 24 h incubation, cell lysates were analyzed by western Blot. The result showed that the protein level of BIKDD was increased by STF treatment compared to the mock treated cells (Figure 13). Next, we investigated whether combined treatment of STF and BIKDD in TNBC cell lines would result in an enhancement in the tumor killing effect. To do this, we performed MTT assay to analyze the cell viability. MTT results showed that although STF treatment alone had little effect on the cell viability, STF combined with BIKDD resulted in a great reduction in the viability of all three TNBC cells (Figure 14). Furthermore, we calculated the combination index (CI). CI of the MTT

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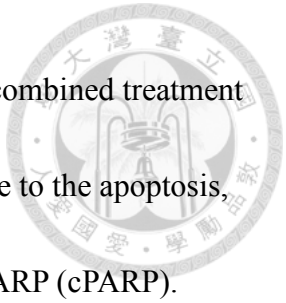

results in three cell lines were all smaller than 1 which indicated the combined treatment has synergism effect. To confirm the decrease of cell viability was due to the apoptosis, we also checked the expression level of apoptosis marker cleavage PARP (cPARP). BIKDD were stably express in TNBC cell lines and STF was treated for 36 h before analyzed by western Blot. cPARP increased significantly in the present of STF compare to the BIKDD only control (Figure15). In summary, we prove the hypothesis that combined treatment of IRE1 inhibitor-STF and BIKDD enhances the killing effect in TNBC cell lines.

Combined treatment of STF and BIKDD enhances the tumor killing effect in the TNBC bearing nude mice

We next tested the combined treatment in a mouse model. To this end, we injected Hs578T cells transiently transfected with BIKDD or control plasmid into the fat pad of nude mice. One week after tumor transplantation, the mice were treated with or without IRE1 inhibitor-STF. The tumor volumes were monitored every three days and the tumors were taken out after 50 days of transplantation. Similar to the *in vitro* tumor killing effect, we showed that STF treatment alone did not significantly affect tumor growth. However, combined administration of STF and BIKDD resulted in a significant reduction in tumor growth, compared with STF or BIKDD treatment alone (Figure 16A,

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B). Additionally, the body weight of mice didn't show significant difference among the four groups (Figure 16C), suggesting lack of toxicity. Thus, combined treatment of STF and BIKDD showed a better tumor killing effect in the mouse model.

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IV. Discussions

In this study, we have discovered an ubiquitin-dependent degradation mechanism for the BH3-only protein BIK. We first showed that knockdown of Cullin5 results in the accumulation of BIK protein. Second, we found that a Cullin5 substrate adaptor ASB11 can promote the ubiquitination of BIK. Third, knockdown of ASB11 increases BIK protein level. These findings collectively indicate that ASB11-based Cullin5 complex is responsible for the ubiquitination and degradation of BIK. Pervious study has reported that BIK can be phosphorylated by ERK1/2 which promotes the ubiquitination and proteasome degradation (*138, 139*). Whether ASB11-based Cullin5 ubiquitin ligase is involved in this ubiquitination process is currently unclear. However, a contrary finding was reported that the increase of BIK expression under ERK1/2 inhibition is a delayed event due to a cell cycle arrest-induced BIK mRNA upregulation, rather than a direct regulation of BIK protein stability (*140*).

After revealing the degradation mechanism of BIK, we further investigated the physiological role and biological function of BIK ubiquitination by ASB11-based Cullin5 complex. First, we found that mRNA and protein of ASB11 can be upregulated under ER stress. Second, ER stress induces a downregulation of BIK and this downregulation is due to ASB11 dependent proteasomal degradation. Third, we showed that ER stress acts through IRE1α/XBP1 pathway to induce *ASB11* transcription.

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Together, these data indicate that IRE1α/XBP1 pathway upregulates ASB11 to degrade BIK under ER stress.

Different phases of ER stress have different strategies for survival or death (*141*). In the beginning of ER stress, cells enter the adaptive phase which temporally maintains the survival under ER stress and cells can take the time to restore ER homeostasis. We propose that the hyper-activation of IRE1α/XBP1 may contribute to the adaptation and survival of cancer cells at this stage of ER stress by degrading pro-apoptotic BIK through the upregulation of ASB11. Knockdown of ASB11 in the adaptive phase under ER stress inducer-tunicamycin increased the bio-marker of apoptosis-cleavage caspase 3 and is reversed by double knockdown of BIK (Appendix 5). This result further support that downregulation of pro-apoptotic protein of Bcl-2 family, BIK, may be a new way that contributes to the survival of adaptive phase under ER stress. After adaptive phase, if cells failed to deal with the ER stress, cells enter chronic ER stress and trigger apoptosis. It has been reported that IRE1/XBP1 is the first pathway that turns off in chronic ER stress, whereas the PERK pathway sustains (*125*). The turn off of IRE1/XBP1 leads to the shutdown of ASB11-based cullin5 complex which indicates BIK will no longer be down-regulated. Additionally, PERK-ATF4-CHOP pathway will express pro-apoptotic protein of Bcl-2 family, for example, Bim which induces apoptosis (*142*). Finally, cells apoptosis and die in the phase of chronic ER stress.

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We show that the IRE1/XBP1 branch of UPR mediates the upregulation of ASB11. Next, we tried to figure out how the IRE1α/XBP1 pathway upregulates ASB11. ChIP analysis revealed that XBP1 is recruited to the *ASB11* promoter. Moreover, by using a set of ChIP primers, we found that XBP1 occupied to a region encompassing -304 to -86 of the *ASB11* promoter. However, out of our expectation, we did not find canonical XBP1 binding motifs in this region. Instead, NF-Y targeting site was identified. NF-Y can form a complex with XBP1, which targets the cis-element called ER stress-response element (ERSE), which is commonly present in the promoter of UPR associated genes. The consensus sequence for ERSE is CCAAT-N9-CCACG (*143*), in which NF-Y and XBP1 target CCAAT and CCAACG, respectively. Thus, the NF-Y-XBP1 complex binds to ERSE in parallel, in other words, next to each other on ERSE. Here, we propose another possibility that XBP1-NF-Y complex can be targeted to the promoter that contains only NF-Y binding site. This possibility explains how XBP1 can be recruited to the *ASB11* promoter which lacks XBP1 targeting site. In support of our hypothesis, we first showed that knockdown of NF-Y blocks the recruitment of XBP1, indicating the requirement of NF-Y for the promoter recruitment of XBP1. Second, luciferase reporter assay in our laboratory also indicates NF-Y conserve binding site is important for the promoter activity (Appendix 6). Furthermore, an interestingly previous study used ChIP-on-chip analysis as an unbiased strategy to identify the promoters bound by

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XBP1 and then performed bioinformatics analysis for predicting the preferred binding site of XBP1 using the ChIP-on chip data. This study revealed that a significant subset of XBP1-bound promoters contains only the NF-Y binding site (*136*). Thus, the new mode of XBP1 promoter targeting discovered in our study likely represents a prevalent mechanism. Notably, it has been reported that NF-Y is involved in cancer progression and the over-expression of NF-Y target genes are related to the poor prognosis (*144*). Here, we hypothesize that NF-Y may induce ASB11 to decrease the pro-apoptotic protein-BIK, thereby promoting cell survival. This mechanism may be another possible reason to explain the pro-tumor function of NF-Y.

After revealing the pathway that regulate the degradation mechanism of BIK. At last we tried to apply our discovery to cancer therapy. The cancer therapy of BIKDD is functional in several cancers including pancreatic, lung and breast. However, cancer cells often face stressful conditions and results in ER stress to active UPR which destabilize BIKDD (*145*). Thus, we thought that the inhibition of ASB11-dependent ubiquitination can enhance the stability and anti-tumor efficacy of BIKDD. The MTT assay of TNBC cell lines and the TNBC bearing mouse model both supported that the combined treatment of IRE inhibitor-STF and BIKDD has a better tumor killing effect. We chose TNBC for our model for several reasons. First, TNBC is the most malignant subtype of breast cancer with limited treatment options. Second, it has been reported

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that IRE1α/XBP1 pathway is highly active in TNBC (*146*), which predicts a reduced stability of BIKDD in this cancer type. Third, XBP1 and HIF1α cooperatively promote the tumorigenesis and progression of TNBC (*134*). Thus, blockage of IRE1α/XBP1 axis may be of additional beneficial effect beyond the stabilization of BIKDD.

Most of the IRE1 inhibitors target the catalytic core including the ATP binding site of the kinase domain and the RNase domain (*137*). There are three groups of inhibitors. The first group inhibits the kinase output by binding to IRE1 kinase domain or by competing with ATP. The second is the quercetin group which can trigger the IRE1 dimerization which enhances the RNase output and lowers the kinase output. The third is the salicylaldehyde group which reversibly and selectively inhibits IRE1 RNase ability with little effect on its kinase activity. The STF-083010 used in our study belongs to the third group. Recently, it has been reported that XBP1 is a synthetic lethal partner of oncogene-*myc* and the new IRE1 inhibitor-8866 belonging to the salicylaldehyde group can suppress the growth of breast cancer with a high MYC expression (*147*). Thereby, it would be important to test whether 8866 in combination with BIKDD can also offer a synergism effect in anti-tumor activity.

One problem for the BIKDD used in our experiments is that the CMV-BIKDD expresses BIKDD non-selectively by the strong promoter-cytomegalovirus (CMV). Global expression of BIKDD could also kill the normal cells and therefore cannot be

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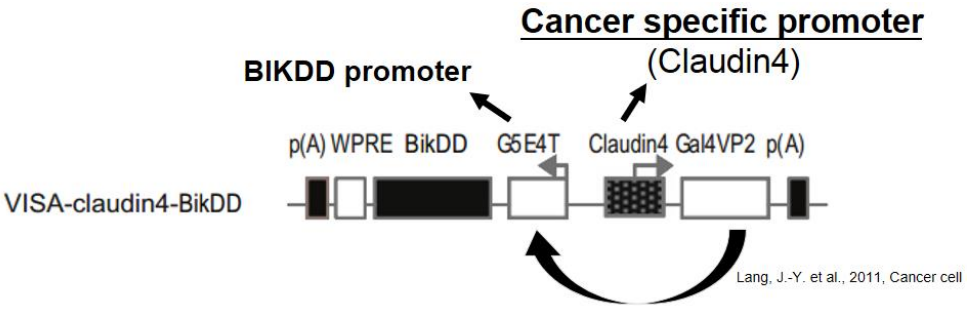
used for gene therapy. To specifically kill the TNBC cells without affecting normal cells, we will try to use the VISA-claudin4-BIKDD (VP16-GAL4-WPRE integrated systemic amplifier) construct (as below). This construct was optimized from two-step transcriptional amplification (TSTA) which utilizes tissue specific antigen to express the BIKDD specifically in the breast cancer cells (*62, 148*). Claudin4 is overexpressed in breast cancer and can serve as a tissue specific antigen. Claudin4 drives claudin4 promoter and expresses the GAL4 fusion protein which drives the expression of BIKDD (*65*). It has been reported that the high level of claudin4 were detected in 66.1% TNBC so that VISA-claudin4-BIKDD is a promising gene therapy for TNBC (*149*).



In this study, we transfected BIKDD into the tumor cells before the tumor transplantation. Although this method is used for an initial test of the concept of combined therapy with BIKDD and IRE1 inhibitor, it is not applicable in the clinics. To improve the feasibility of our combined treatment, we will deliver BIKDD into tumor cells after tumor transplantation and growth in nude mice. The efficient and specific delivery of BIKDD to tumors is the most critical part of gene therapy. Two strategies

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will be tested in the future. First, we will directly inject VISA-BIKDD into the tumor of nude mice by the *in vivo* DNA transfection reagent-linear polyethylenimine (*150*). It has been reported that liposome coded with polyethylene glycol (PEG) can increase the circulation time and prevent liposome clearance of reticuloendothelial system (RES) system (*151*). Another delivery strategy is the utilization of adeno-associated viral (AAV) carrying VISA-BIKDD, which can be injected directly to tail vein. AAV vectors are non-pathogenic, integrating DNA vectors which have the ability to attach broad range of host cell and enter the nucleus. AAV system can stably express gene for a period of time, without the risk of insertional mutagenesis and lack of toxicity (*152*). Future study will be conducted for testing these two strategies of gene therapy.

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VI. Figures

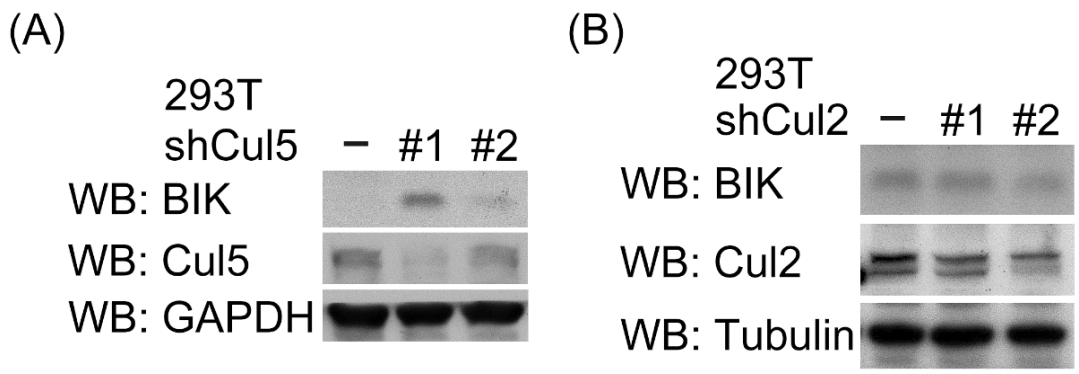

Figure 1. Knockdown of Cul5 but not Cul2 increases the protein level of BIK

(A, B) 293T cells were infected with lentivirus carrying Cul5 shRNAs, Cul2 shRNAs or control shRNA as indicated. The cell lysates were analyzed by western blot with specific antibodies as indicated.

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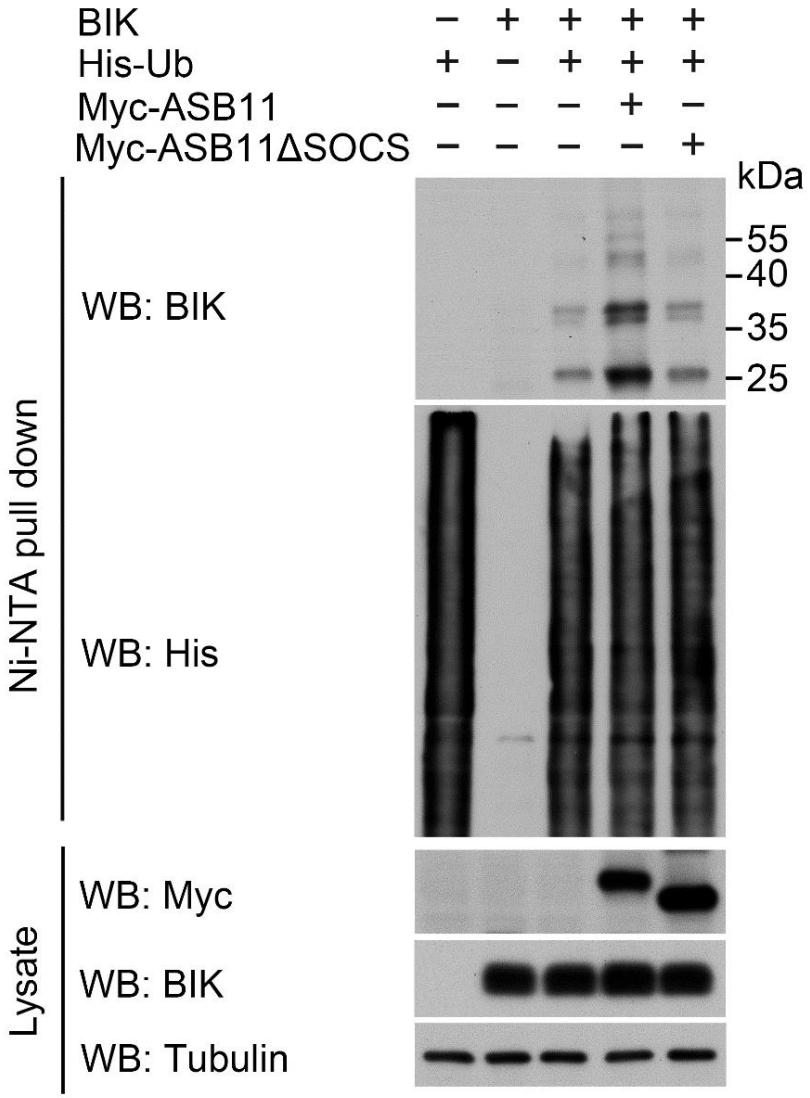

Figure 2. Overexpression of ASB11 but not ASB11 mutant promotes BIK ubiquitination

293T cells were transfected with BIK, His-Ub, Myc-ASB11 wild type or Myc-ASB11 mutant as indicated. Cells were treated with 1 μM proteasome inhibitor MG132 and ubiquitinated BIK was pulled-down under denaturing conditions by Ni-NTA beads and analyzed by western blot.

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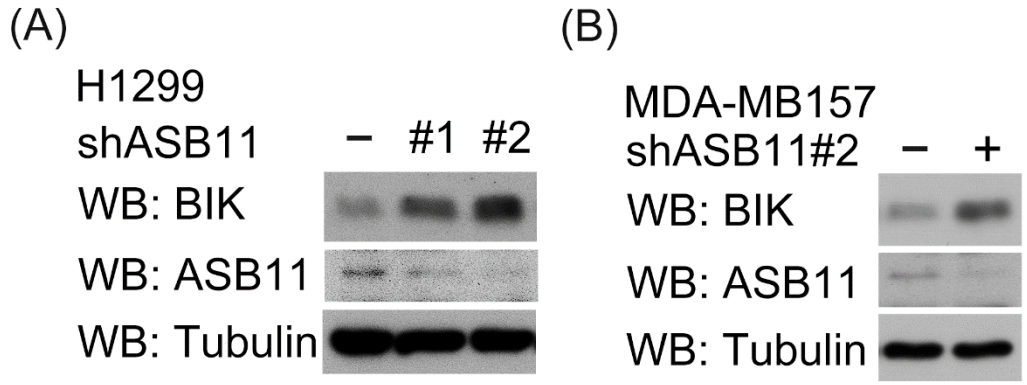

Figure 3. Knockdown of ASB11 increases the protein level of BIK in H1299 and MDA-MB 157

(A, B) H1299 or MDA-MB157 were infected with lentivirus carrying ASB11 shRNAs or control shRNA as indicated. Cell lysates were analyzed by western blot with specific antibodies as indicated.

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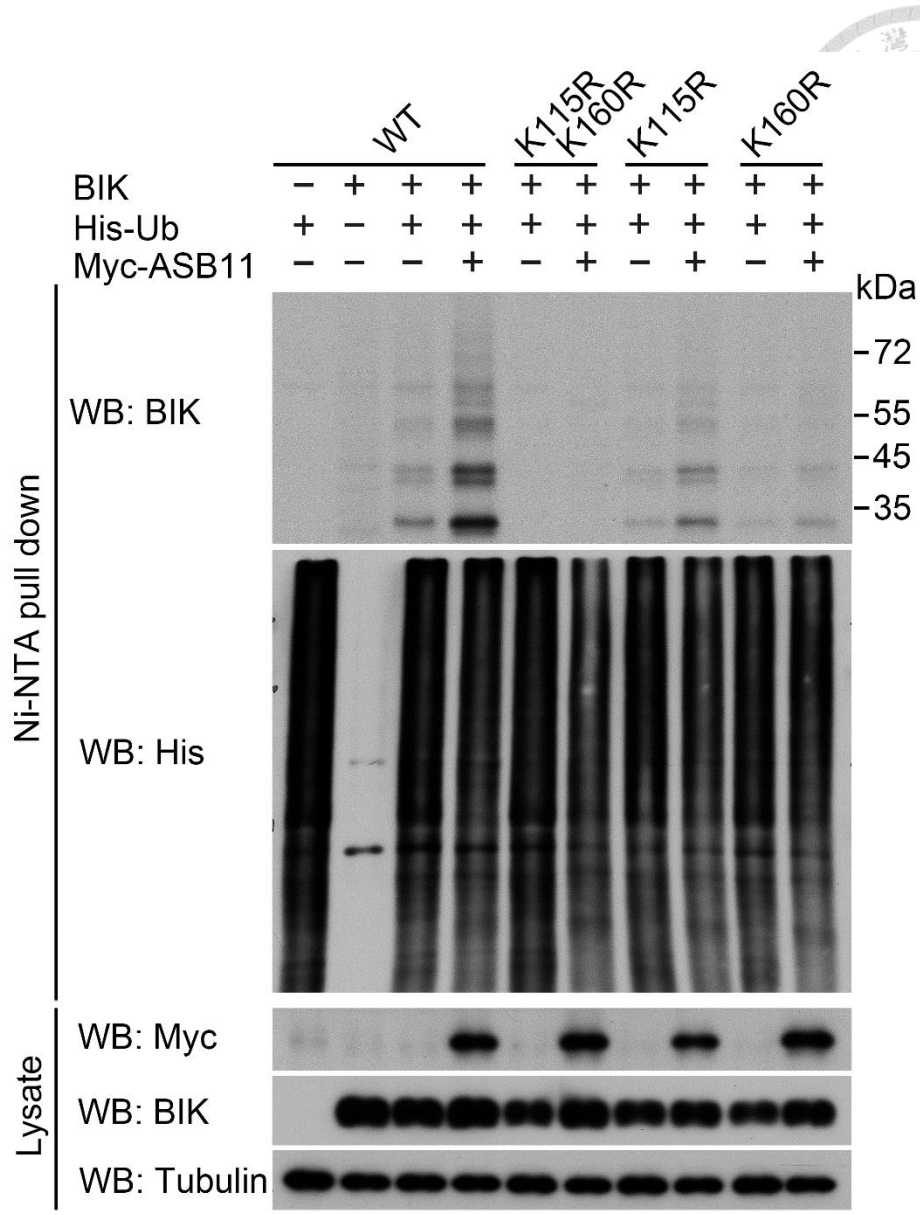

Figure 4. ASB11 promotes BIK ubiquitination at lysine115 and lysine160

293T cells were transfected with His-Ub, Myc-ASB11, BIK wild type or various K-to-R mutants of BIK as indicated. Cells were treated with 1 μM proteasome inhibitor MG132 and ubiquitinated BIK was pulled-down under denaturing conditions by Ni-NTA beads and analyzed by western blot.

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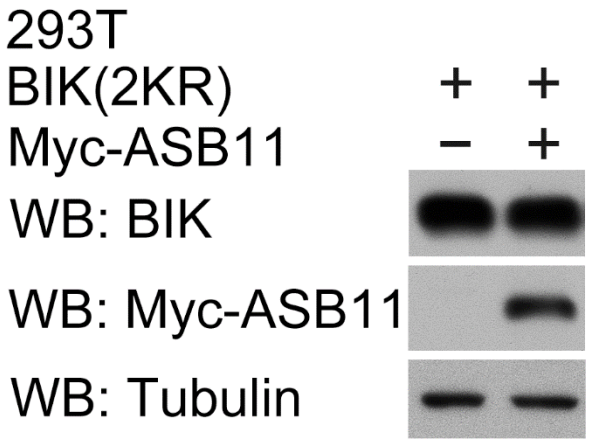

Figure 5. ASB11 cannot promote the degradation of BIK(2KR) mutant

293T cell were transfected with BIK(2KR) mutant and Myc-ASB11 as indicated. Cell lysates were analyzed by western blot with specific antibodies as indicated.

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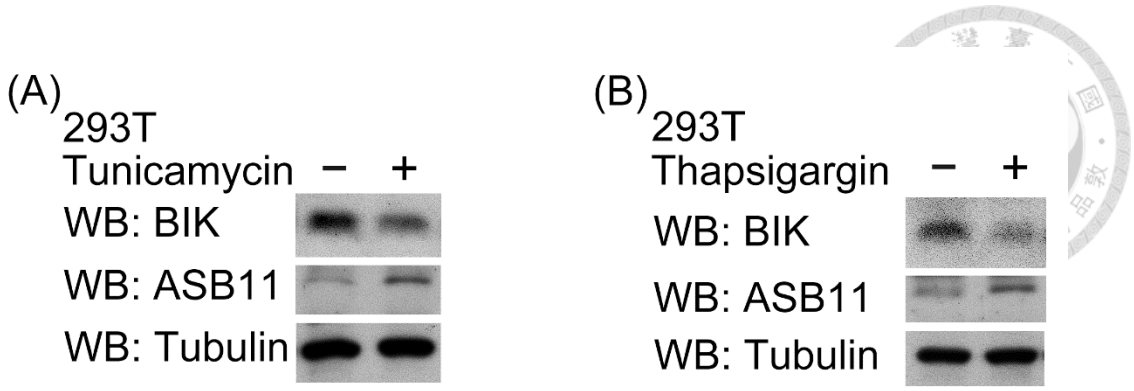

Figure 6. ER stress inducers upregulate ASB11 protein to decrease BIK expression

(A, B) 293T cells were treated with ER stress inducers 10 μg/ml tunicamycin (Tu) or 500 nM thapsigargin (Tg) as indicated for 16 h. Cell lysates were analyzed by western blot with specific antibodies as indicated.

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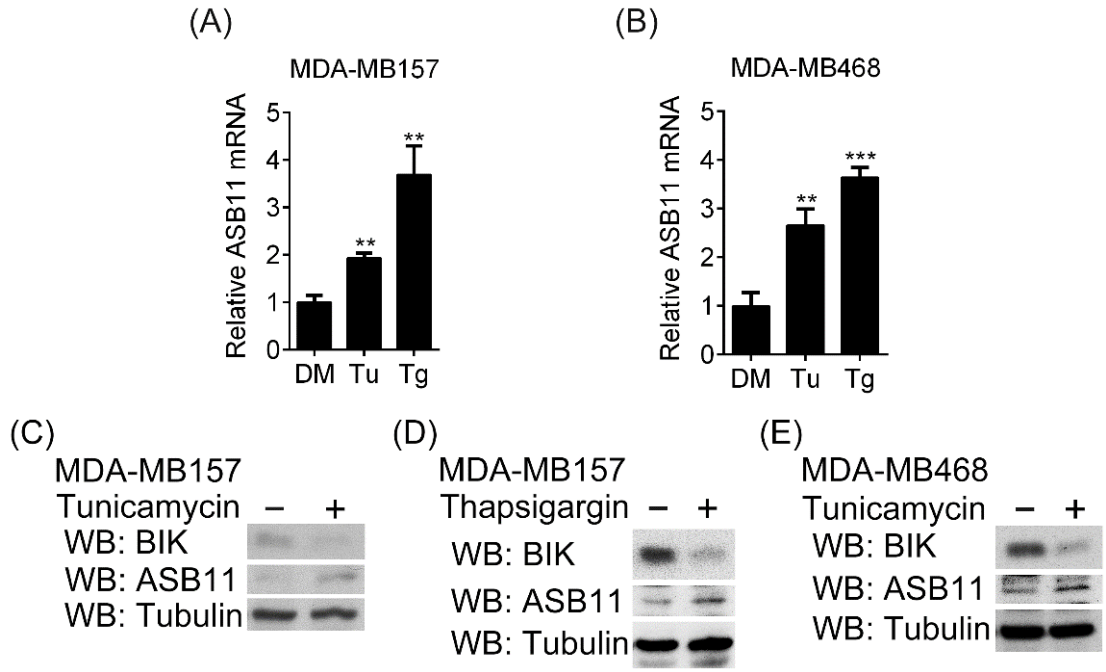

Figure 7. ER stress inducers can upregulate mRNA and protein levels of ASB11 and decrease BIK

(A, B) TNBC cell lines as indicated were treated with ER stress inducers 10 μg/ml tunicamycin or 500 nM thapsigargin for 8 h. Cells were harvested for RT-qPCR analysis of *ASB11* mRNA expression. Data represent the mean ± S.D., n=3, **<P0.01 and ***P<0.001 by t-test. (C-E) TNBC cell lines as indicated were treated as (A, B). Cell lysates were analyzed by western blot with specific antibodies as indicated.

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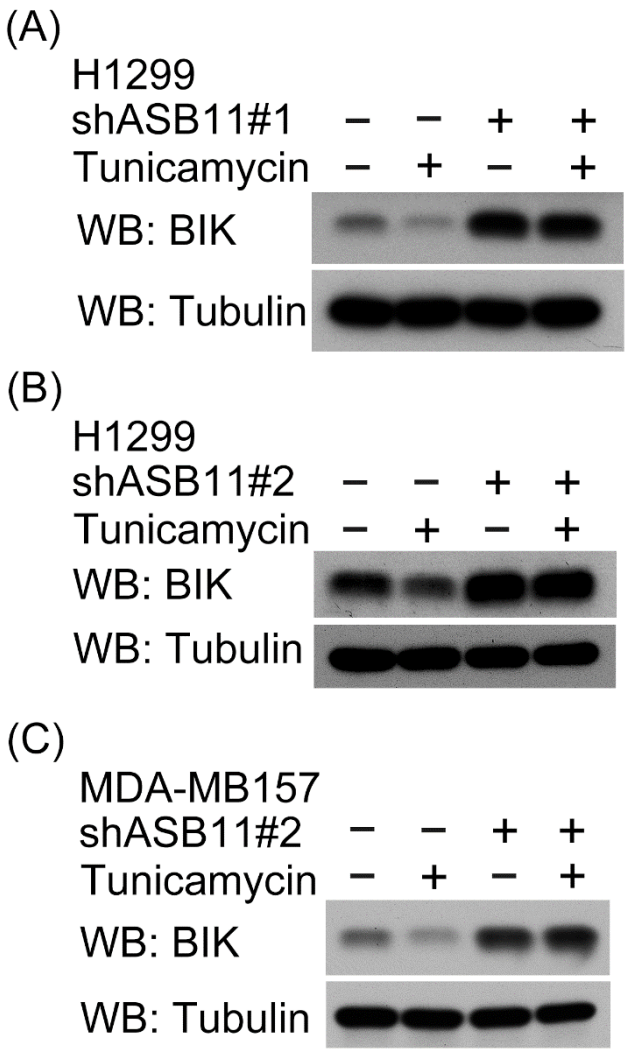

Figure 8. ER stress inducer decreases BIK through ASB11

(A-C) H1299 or MDA-MB157 cell lines as indicated were infected with lentivirus carrying ASB11 shRNAs or control shRNA. Cells were treated with ER stress inducer 10 μg/ml tunicamycin for 8 h as indicated. Knockdown efficiencies of both cell lines were shown in Figure 3.

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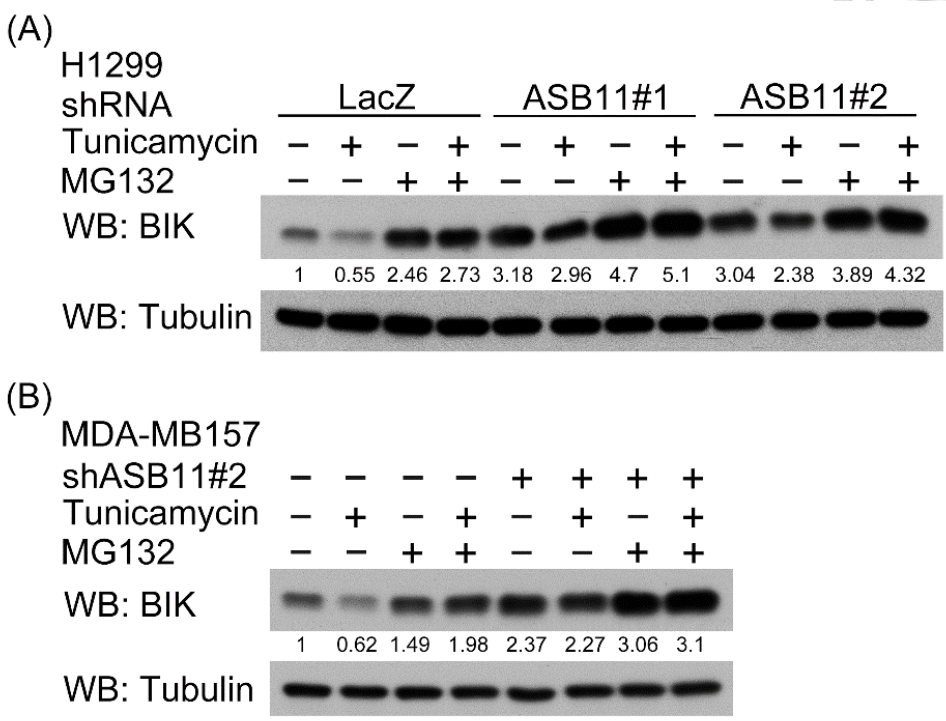

Figure 9. ER stress inducer decreases BIK by proteasome degradation through ASB11

(A, B) H1299 or MDA-MB157 cells were infected with lentivirus carrying ASB11 shRNAs or control shRNA. Cells were treated with ER stress inducer 10 μg/ml tunicamycin and 500 nM proteasome inhibitor MG132 for 8 h. Cell lysates were analyzed by western blot with specific antibodies as indicated. The amount of BIK was quantified and normalized to the amount of tubulin, and the ratio was shown below. Knockdown efficiencies of both cell lines were shown in Figure 3.

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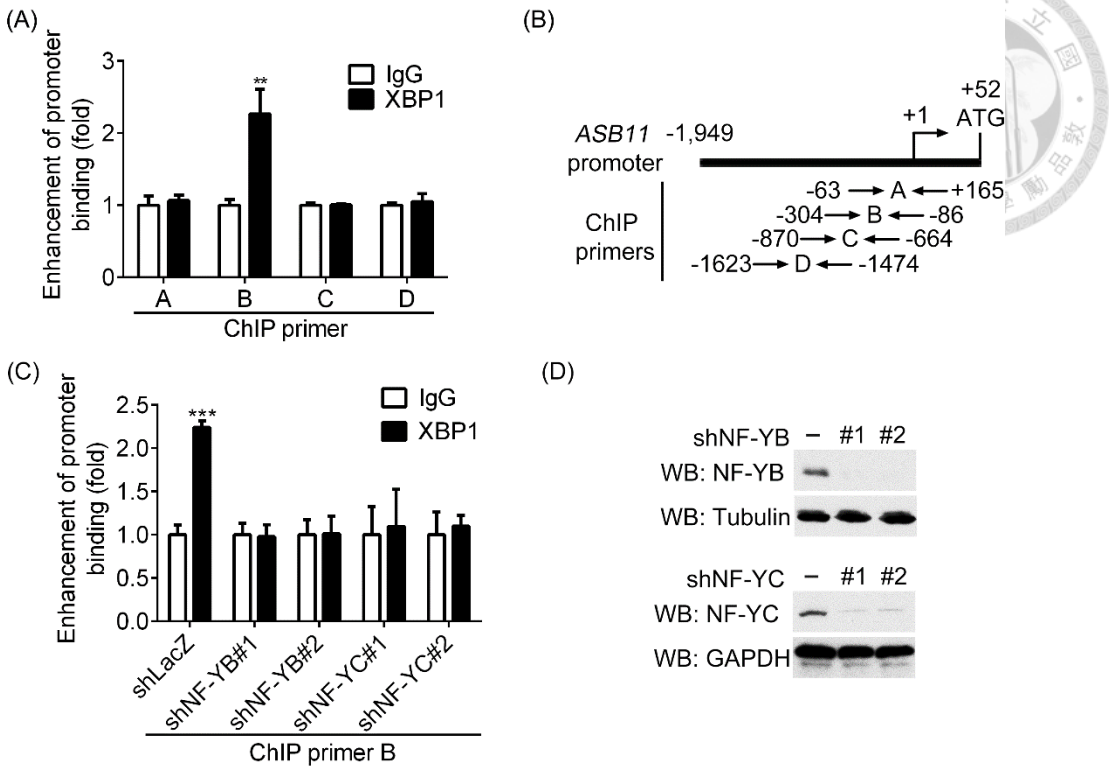

(Collaborate with Fei-Yun Chen)

Figure 10. XBP1 is recruited to *ASB11* promoter

(A) 293T cells were treated with ER stress inducer 10 μg/ml tunicamycin for 4 h and then harvested. Cell lysates were sonicated *and* immunoprecipitated *with I*gG or XBP1 antibodies by magnetic beads overnight. Total DNA were extracted from the immunoprecipitates and then analyzed by qPCR with 4 pairs of chromatin immunoprecipitation (ChIP) primers as indicated. Data represent the mean ± S.D., n=3, **P<0.01 and ***P<0.001 compare to the control IgG by t-test. (B) The promoter region of *ASB11* was divided into four regions A, B, C, D as indicated. (C) 293T cells were infected with lentivirus as indicated. ChIP assay was performed as (A). (D) The knockdown efficiency of NF-YB and NF-YC.

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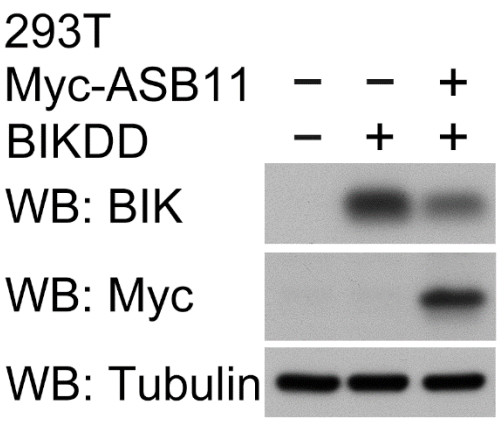

Figure 11. ASB11 downregulates BIKDD

293T cells were transfected with Myc-ASB11 and BIKDD. Cell lysates were analyzed by western blot with specific antibodies as indicated.

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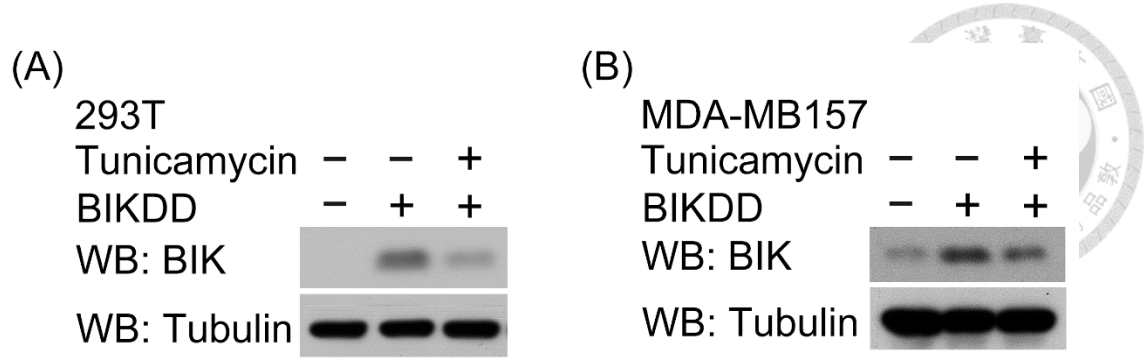

Figure 12. ER stress inducer downregulates BIKDD

(A, B) 293T and MDA-MB157 cells were transfected with BIKDD and treated with ER stress inducer 10 μg/ml tunicamycin for 8 h. Cell lysates were analyzed by western blot with specific antibodies as indicated.

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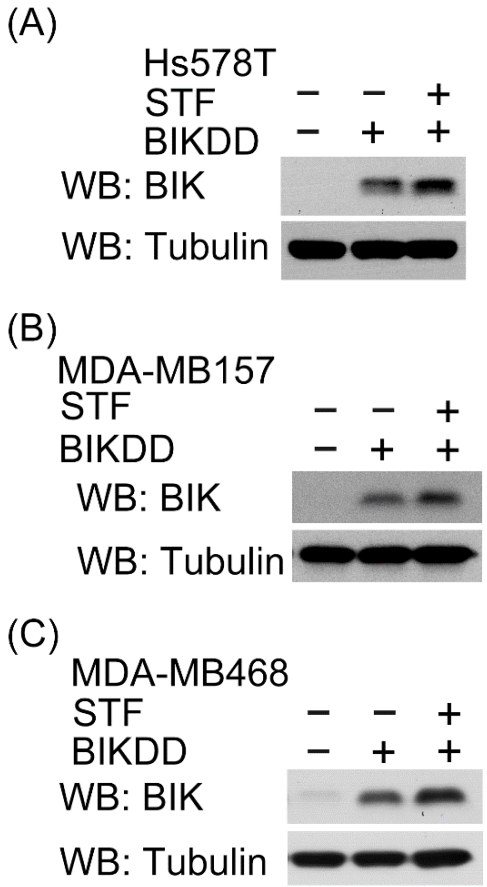

Figure 13. IRE1 inhibitor stabilizes BIKDD in three TNBC cell lines

(A-C) TNBC cell lines as indicated were infected by the virus which stably expressed BIKDD. Cells were treated with IRE1 inhibitor 100 μM STF-083010 for 24 h. Cell lysates were analyzed by western blot with specific antibodies as indicated.

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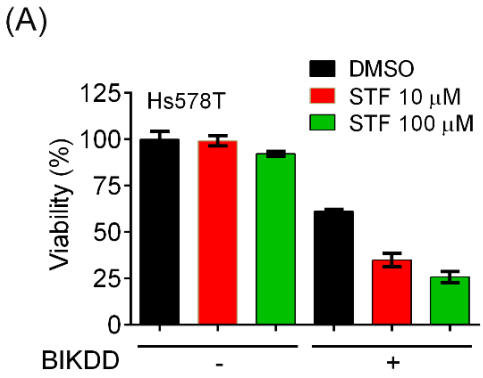
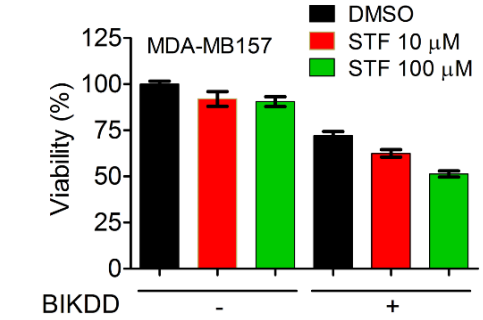
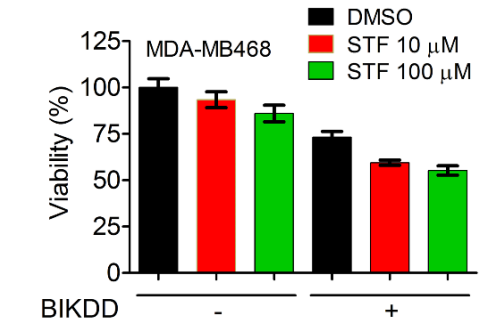


	BIK	
STF	10	100
CI	0.61	0.63



	BIK	
STF	10	100
CI	0.79	0.7



	BIK	
STF	10	100
CI	0.71	0.89

(Collaborate with Fei-Yun Chen)

Figure 14. Combined treatment of IRE1 inhibitor and BIKDD enhances the killing of TNBC cell lines

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(A-C) TNBC cell lines as indicated were transfected with CMV-BIKDD one day before treated with different concentration of IRE1 inhibitor STF-083010 or DMSO as indicated for 48 h. The viabilities of cells were analyzed by the MTT assay. The data represent the mean ± S.D. and the combination index (CI) is indicated below.

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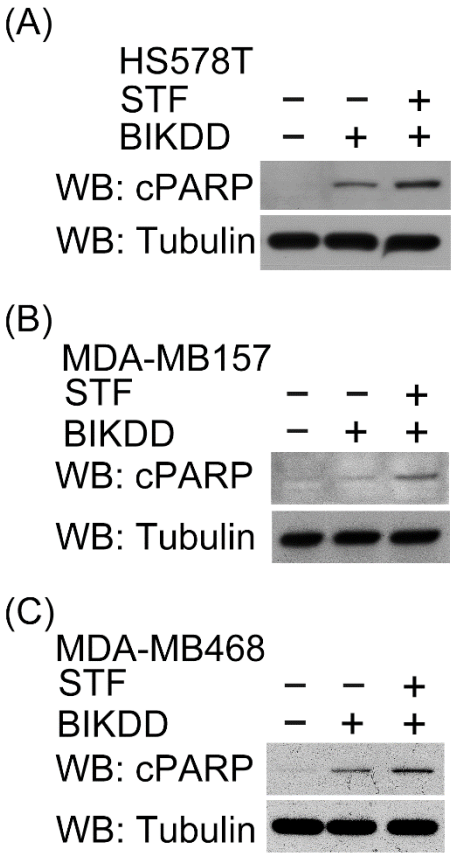

Figure 15. IRE1 inhibitor increases the cleavage PARP in three TNBC cell lines

(A-C) TNBC cell lines as indicated were infected by the virus stably expressing BIKDD. Cells were treated with IRE1 inhibitor 100 μM STF-083010 for 36 h. Cell lysates were analyzed by western blot with specific antibodies as indicated.

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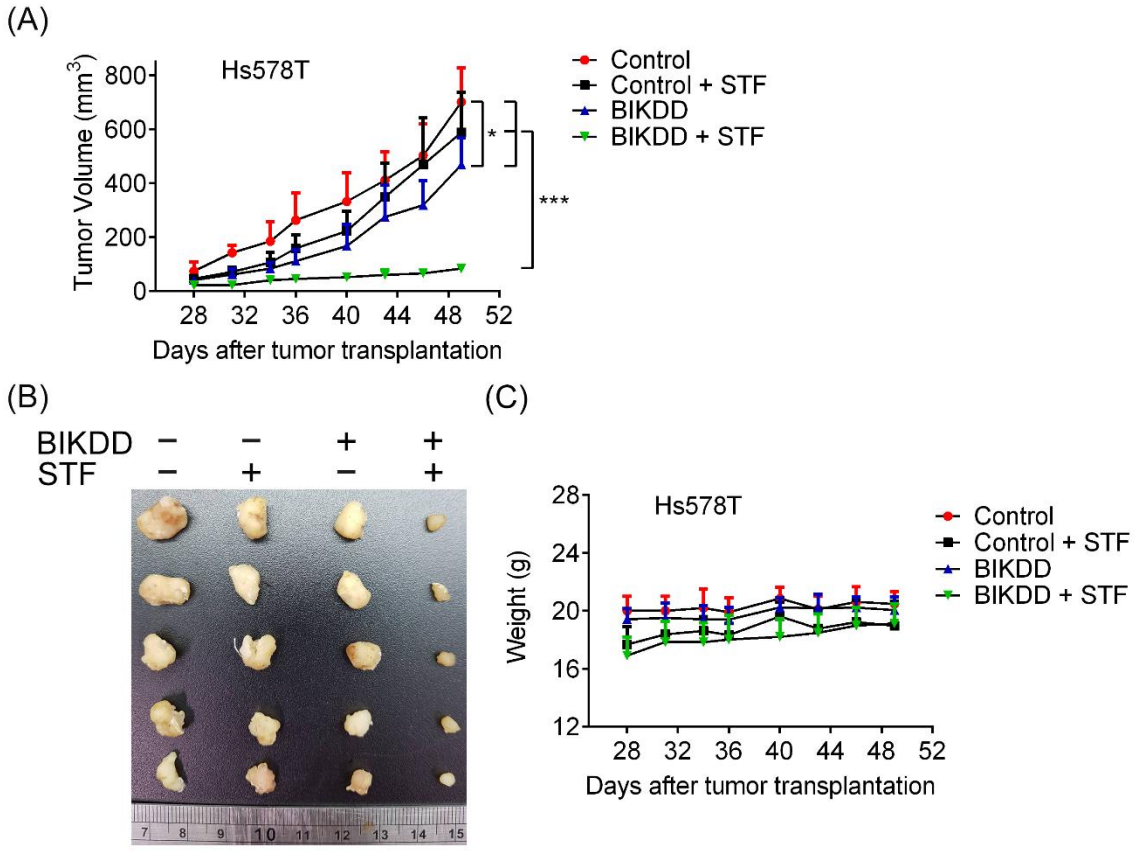

(Collaborate with Fei-Yun Chen)

Figure 16. Combined treatment of IRE1 inhibitor and BIKDD enhances the killing effect of BIKDD in TNBC bearing nude mice

(A, C) Hs578T cells were transfected with control vector or BIKDD as indicated. Hs578T cells were counted and $2*10^6$ cells were injected into the fat pad of female nude mice. Seven days after tumor transplantation, DMSO or 40 mg/kg IRE1 inhibitor STF-083010 were intraperitoneal injected every three days. The tumor growth and the weight were measured at 28 days after tumor transplantation. Data represent the mean ± S.D. (n=5 per group), *P<0.05 and ***P<0.001 compare to the control by t-test. (B) Images of the tumors taken at 50 days after tumor transplantation.

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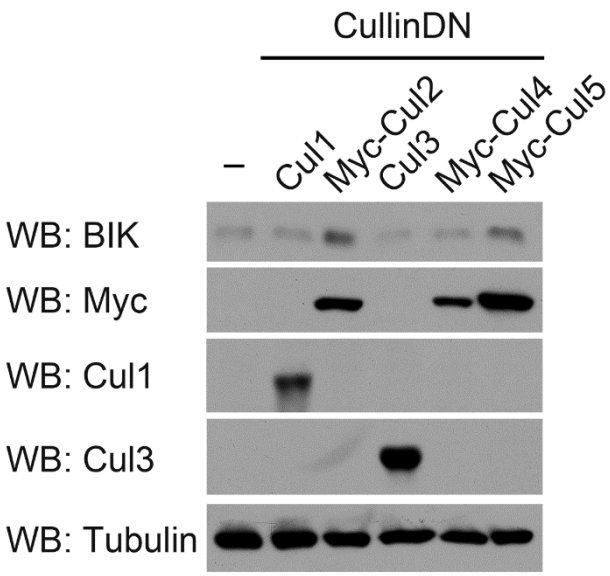
VII. Appendix



(by Fei-Yun Chen)

Appendix 1. Domain negative form of Cul2 and Cul5 upregulate BIK protein

293T cells expressed domain negative form Cullin as indicated. Cell lysates were analyzed by western blot with specific antibodies as indicated.

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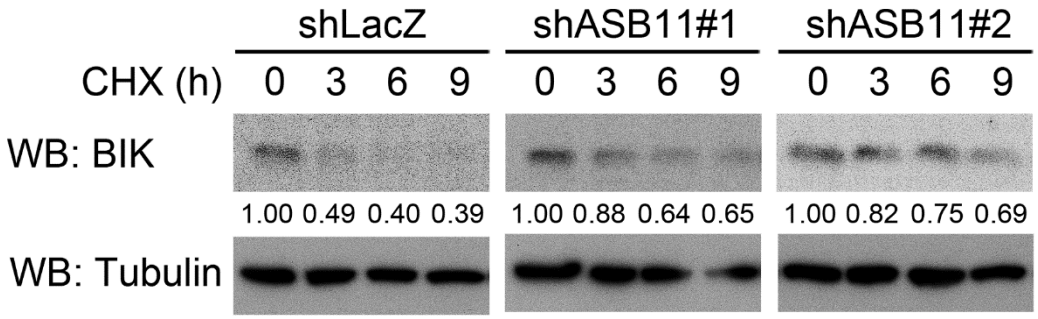

(by Fei-Yun Chen)

Appendix 2. Knockdown ASB11 increase BIK half-life

293T cells were infected with lentivirus carrying ASB11 shRNAs or control shRNA. Cells were treated with 50 μg/ml cycloheximide for indicated time points before harvest for western blot analysis.

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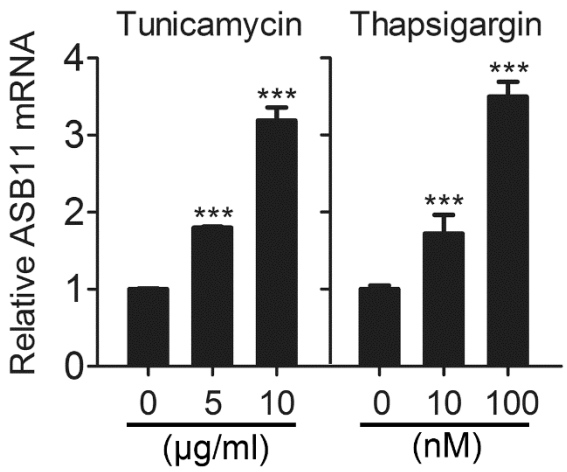

Appendix 3. ER stress inducer can upregulate ASB11 mRNA in 293T

293T cells were treated with ER stress inducers tunicamycin or thapsigargin as indicated concentration for 16 h. Cells were harvested for RT-qPCR analysis of *ASB11* mRNA expression. Data represent the mean ± S.D., n=3 and ***P<0.001 by t-test.

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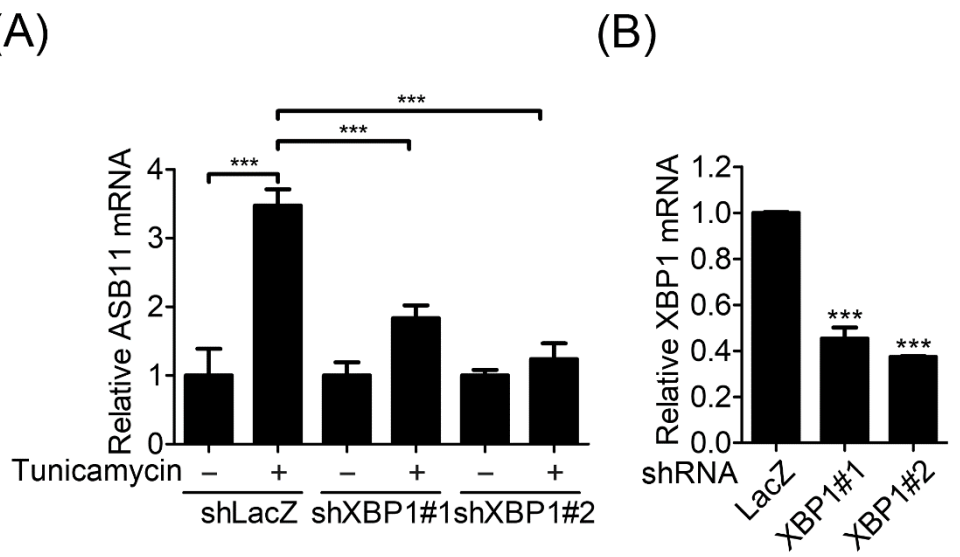

(by Fei-Yun Chen)

Appendix 4. ER stress inducer increases *ASB11* mRNA expression through XBP1

(A) 293T cells were infected with lentivirus carrying XBP1 shRNAs or control shRNA. Cells were treated with 10 μg/ml tunicamycin for 16 h and then harvested for RT-qPCR analysis of *ASB11* mRNA expression. Data represent the mean ± S.D., n=3 and ***P<0.001 by t-test. (B) The knockdown efficiencies of XBP1 shRNAs.

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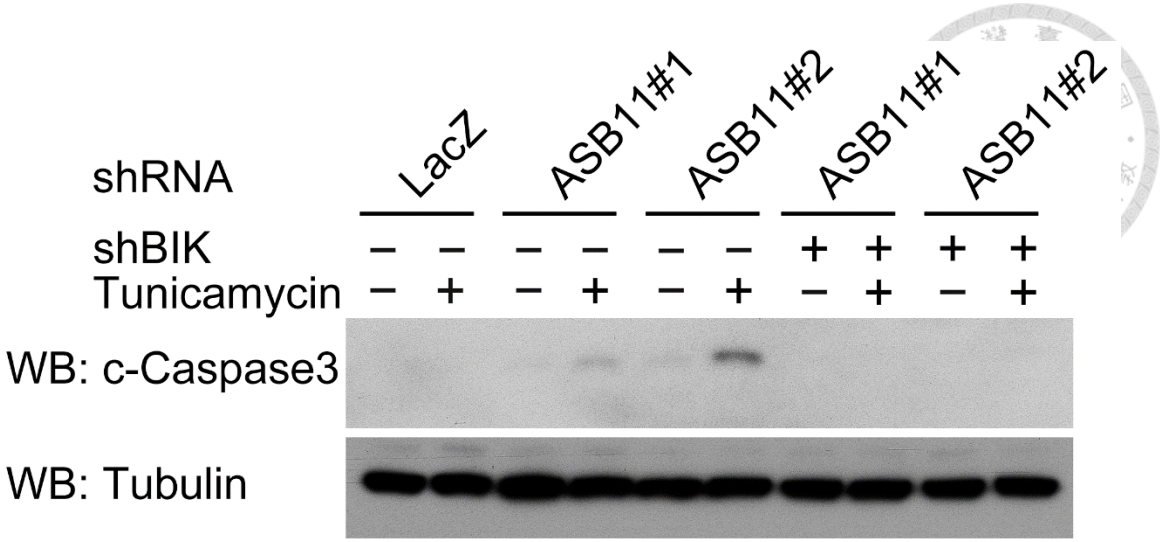

(by Fei-Yun Chen)

Appendix 5. ASB11 knockdown induces apoptosis under adaptive response of ER stress and reverse by BIK knockdown

293T cells were infected with lentivirus carrying ASB11 shRNAs or control shRNA and treated with 10 μg/ml tunicamycin for 18 h before harvest for western blot analysis.

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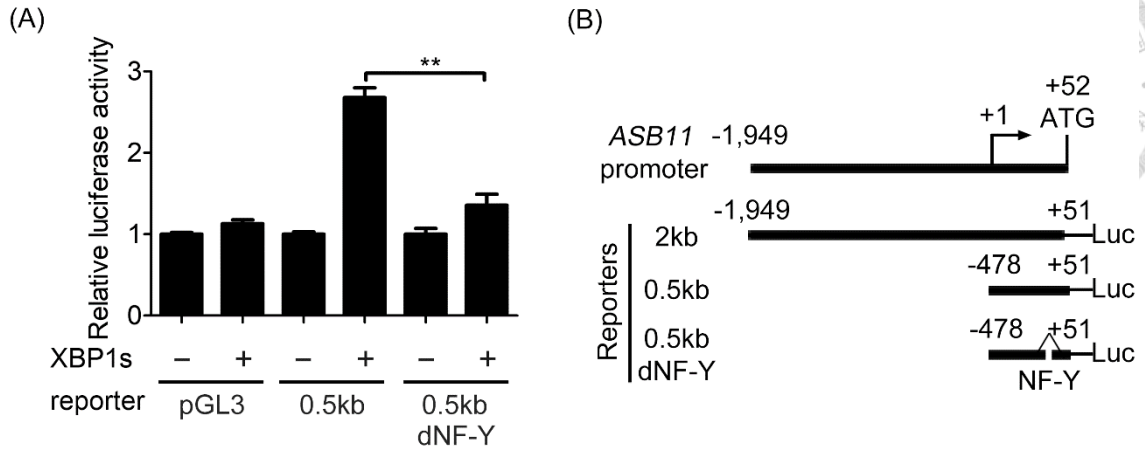

(by Fei-Yun Chen)

Appendix 6. NF-Y conserve binding site is critical for the promoter activity of *ASB11*

(A) Luciferase reporter assay of 293T cells transfected with XBP1s or control vector together with the indicated reporter constructs. Data represent the mean ± S.D., n=3, $^{**}P<0.01$, by one-way ANOVA with Turkey's post test (B) The promoter region of *ASB11* was cloned into reporter gene with or without NF-Y conserve binding site as indicated.

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