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國家衛生研究院整合性醫藥衛生科技研究計畫

探索E1A在抑制腫瘤發展及癌轉移之分子機制

計畫名稱

97年度成果報告

執 行 機 構： 中國醫藥大學附設醫院

計 畫 主 持 人： 蘇振良 副研究員

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目錄

壹、97年度計畫研究成果摘要

計 畫 名 稱：探索E1A在抑制腫瘤發展及癌轉移之分子機制

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計畫主持人：蘇振良

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關 鍵 字：乳癌,癌轉移,腺病毒蛋白type5 E1A, 訊息傳遞, PP2A去磷酸脢, 發炎反應, 上皮向間質轉化(EMT)

成 果 分 類：
- ☑ 癌症基礎與臨床研究(可複選，最多三項)
- ☑ 分子與基因醫學研究
- ☐ 臨床研究
- ☐ 生物技術與藥物研究
- ☐ 生物統計與生物資訊研究
- ☐ 醫療保健政策研究
- ☐ 環境衛生與職業醫學研究
- ☐ 醫學工程研究
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- ☐ 幹細胞研究
- ☐ 奈米醫學研究
- ☐ 其他重要疾病或醫藥衛生問題研究

中文摘要

乳癌是目前世界上女性癌症病患中為數最多的癌症之一，因此針對乳癌發展出嶄新的方式用以診斷、預防以及治療有其迫切的需求，將基礎研究的成果應用到臨床上以增進癌症病人的療效則是其中一項達成此目標的重要方式。報導已知第五型腺病毒蛋白(type5 E1A) 和許多抗腫瘤作用有關，目前於多種癌症如乳癌、卵巢癌及頭頸癌之病患進行臨床上第二期之基因治療試驗。因此，了解與 E1A 相關之抗腫瘤作用中詳細之分子機制是相當重要，為了達到此目的，我們將著重於 E1A 所參與之訊息傳遞作用及 E1A 對於腫瘤生長的抑制作用，我們已發現多種可能與 E1A 抗腫瘤活性相關之作用，其中 E1A 在不同種類的癌細胞中會增加癌細胞對於抗癌藥物的敏感度，例如:在乳癌及卵巢癌癌細胞中 E1A 會增強紫杉醇所引起的細胞凋亡。而我們發現 Forkhead box O-class (FOXO)轉錄因子 FOXO3a 在其中扮演十分重要的角色，利用 RNA 干擾技術去抑制 FOXO3a 的表現則可以有效減弱 E1A 所調節癌細胞對於紫杉醇的藥物敏感度，其中 E1A 穩定 FOXO3a 的表現是經由抑制泛素降解系統: β-transducin repeat–containing proteins (βTrCP) 是 E3 泛素連接酶與穩定 FOXO3a 的表現有相當關係，而當 FOXO3a 被 IKKβ 磷酸化在 Ser644 的位置之後，βTrCP 則會因此與 FOXO3a 結合，進而透過泛素降解系統將 FOXO3a 降解，而 E1A 則可以透過抑制 IKKβ 的活性去達到抑制 βTrCP 對於 FOXO3a 所調節的泛素降解路徑。更進一步，我們發現 E1A 抑制 IKKβ 的活性是透過 protein phosphatae 2A(PP2A)這個絲氨酸及蘇氨酸去磷酸酶來進行調控，因為 E1A 會引起 PP2A 的表現量增加，增加的 PP2A 會與 transforming growth factor β–activated kinase 1 (TAK1)結合，因此降低 TAK1 活性進而抑制 TGF-β 訊息傳遞路徑，使得 IKKβ 的活性也因此被抑制。這樣的結果提供了一個合理的解釋對於之後臨床應用上去合併

E1A 基因治療與紫杉醇化學治療，同時也將在設計標靶治療上提供一道新的曙光。

FOXO3a 對於 E1A 調控之化療藥物增敏作用極具影響

E1A 基因治療在許多不同的化療藥物裡，都有增加其化療藥物增敏作用。例如在治療乳癌和卵巢癌的化療藥物裡：palclitaxel，*E1A* 基因治療有增加其化療藥物增敏作用（Liao et al., 2007）。同時在其他文獻也指出，具有 palclitaxel 的耐受性細胞株裡表現低含量的 FOXO3a（Sunters et al., 2003），於是我們提出說是否 FOXO3a 對於 E1A 調控之化療藥物增敏作用會造成影響。在本報告中，我們利用不同的癌症細胞來看 FOXO3a 對於 E1A 的影響，同時我們在 NIH3T3 纖維母細胞中也發現 FOXO3a 在帶有 E1A 的細胞裡有明顯地表現增加（Figure 1A）。更重要的是，我們利用 RNAi 技術使得 FOXO3a 不表現(siFOXO3a)，發現說 E1A 調控之化療藥物(palclitaxel)增敏作用在 MDA-MB-231 乳癌細胞和 SKOV3-ip1 卵巢癌細胞在轉染 siFOXO3a 細胞中有不見地現象產生（Figure 1B）。我們也利用西方墨點法看 siFOXO3a 的表現（Figure 1C）。我們也更進一步使用動物模型來看 FOXO3a 對於 E1A 調控之化療藥物增敏作用，此動物經原位注射上列之細胞株。結果顯示出 E1A 調控之化療藥物（palclitaxel）的增敏作用在動物模型裡亦有顯現。結果顯示出在 231 細胞中表現 E1A 基因和 231 細胞轉殖小鼠裡同時用 palclitaxel 治療，231 細胞中表現 E1A 基因的轉殖小鼠腫瘤體積

有明顯地下降（在 Figure 1D，810.7±73.2mm^3（231/E1A，lane6）和 1648.7±237.4mm^3（231/vc，lane2）比較）。更重要地是，當我們利用 RNAi 技術使得 FOXO3a 不表現時，在 231/E1A 細胞中可以發現 E1A 調控之 palclitaxel 增敏作用有不見地現象產生（在 Figure 1D，810.7±73.2mm^3（lane6）和 1855.1±135.8mm^3（lane8）比較。在 Figure 1E 中，siFOXO3a 處理過的 231/E1A 會減少 FOXO3a 的表現可能會造成腫瘤體積增加。所以我們做一個結論是 FOXO3a 對於 E1A 調控之化療藥物（palclitaxel）的增敏作用扮演著非常重要的角色。

E1A 防止 FOXO3a 被蛋白酶分解

轉譯後修飾和調節 FOXO3a 的穩定度對於 FOXO3a 的活性很重要（Hu et al., 2004）。因此我們在表現 E1A 的乳癌細胞中探討 FOXO3a 的穩定度。我們使用 231/vc 和轉染 E1A 到 231 細胞（231/E1A）來探討，並且同時添加了 cycloheximide，再依不同時間來阻斷 de novo 蛋白合成。發現說在超過七小時後，FOXO3a 半衰期在 231/E1A 細胞裡表現來得比較多，在少於 1.5 小時，可發現 FOXO3a 半衰期有減少地趨勢。（Figure 2A，使用西方墨點法分析）。TNF-α 調節的 FOXO3a 降解作用（Hu et al., 2004）在 231/E1A 和 231/vc 比較後有明顯的下降（Figure 2B）結果顯示出 E1A 可能經由防止蛋白酶分解 FOXO3a 來增加 FOXO3a 表現。

βTrCP 參與 E1A 誘導 FOXO3a 的表現

為了要探討 TNF-α 調節的 FOXO3a 降解作用是否經由 Ub E3 ligase。我們發現 TNF-α 活化 IKK 去磷酸化 FOXO3a 在 Serine 644 的位置(Hu et al., 2004)，而且在 Serine 644 的位置附近含有一段序列跟 βTrCP 所認之序列相同。於是我們提出 βTrCP 可能參與 FOXO3a 降解。在這個報告中，我們要探討 βTrCP 是否參與 FOXO3a 降解作用，所以我們使用蛋白酶抑制劑 MG132 分別加入 231/vc 和 231/E1A 細胞裡，並且利用免疫沈澱法和西方墨點法來看 βTrCP 是否會跟 FOXO3a 作用。結果顯示出內生性的 FOXO3a 和內生性的 βTrCP 可能在 231/vc 細胞中有交互作用，且這個反應是被 TNF-α 所誘發地。有趣地是，我們發現在 231/E1A 中 TNF-α 誘發地 βTrCP 和 FOXO3a 結合作用有下降的現象。(Figure 3A)。這個現象我們使用接上 Tag 的 HA-FOXO3a 和 Myc-βTrCP 一起轉染到 HeLa 細胞裡，並且利用 HA 和 Myc 的抗體來確認。(Figure 3B)。βTrCP 的降解區域(F-box)提供了 βTrCP 降解能力，所以我們將降解區域（F-box）刪除掉（Myc-βTrCPΔF），發現不會和 FOXO3a 作用（Figure 3C)。此外我們也利用 RNAi 技術使得 βTrCP 不表現(siβTrCP)顯示出 βTrCP 對於 FOXO3a 表現很重要。同時轉染 βTrCP1 和 βTrCP2 增加的 FOXO3a 表現會比單一轉染 βTrCP1 或 βTrCP2 還要好（Figure 3D，lane4 和 2,3 比較)。同時 siβTrCP 會使得 βTrCP 不去降解

FOXO3a（Figure 3E）。此外，在表現 E1A 細胞裡發現 βTrCP 不去降解 FOXO3a（Figure 3D），於是我們作個結論，這些結果顯示出 βTrCP1 和 βTrCP2 參與了 FOXO3a 降解作用，而 E1A 會抑制 βTrCP 去降解 FOXO3a。

E1A 經由抑制 IKKβ 調節 FOXO3a 在絲胺酸 644 的位置磷酸化和防止 βTrCP 誘發 FOXO3a 降解

我們知道 βTrCP 認磷酸化位置是屬於 Serine/Threonine 這一類，並且使得 TNF-α 活化 IKK 去磷酸化 FOXO3a 在 Serine644 的位置(Hu et al., 2004)。為了要證實這一點，我們利用動物實驗發現 FOXO3a 在過度表現 IKKβ 的 Hela 細胞裡對於 βTrCP 表現有明顯增加（Figure 4A），此外，我們利用將磷酸化位置作突變（GFP-FOXo3a/S644E），發現跟 wild type 比較起來和 βTrCP 作用更強（Figure 4B）。FOXO3a 目前已知會被 Akt 磷酸化水解在三個不同的位置上（Thr32,Ser253,Ser315）(Brunet et al.,1999)。此外，這些 Akt 磷酸化的位置不會和 βTrCP 作用（Figure 4B）。

我們利用 FOXO3a 胜肽（包含磷酸化 serine 644 的位置）來看說是否磷酸化 FOXO3a 會跟 βTrCP 結合，結果顯示和控制組比較並無此現象產生（Figure 4C）。下一個我們為了要確認 IKKβ 是否跟 βTrCP 結合 FOXO3a 有關聯性，於是使用接上 Tag 的 HA-FOXO3a 和 Myc-βTrCP 一起轉染到具有缺失的 IKKβ 老鼠胚胎細胞（MEF）來檢視它們對於 IKKβ 是否有影響。而

且當 IKKβ 被重新表現後，βTrCP 對於 FOXO3a 結合的能力又再次出現。顯示出 IKKβ 所調控的磷酸化 FOXO3a 在 Ser644 的位置是 βTrCP 的結合位置。

為了確認 E1A 調節 FOXO3a 穩定度可防止 FOXO3a 被 IKKβ 磷酸化在 serine 644 的位置，且會被 βTrCP 所辨認。我們利用 231/VC 和 231/E1A 兩株細胞來看說 FOXO3a 磷酸化在 Serine 644 的位置對於 FOXO3a 和 βTrCP 的影響。有趣地是，在 231/E1A 中我們發現 TNF-α 調節 FOXO3a 磷酸化在 Serine 644 的位置有明顯下降的現象（Figure 4E）。此外，在 231/E1A 中，TNF-α 調節 FOXO3a 對於 βTrCP 結合也是下降的（Figure 4E）。在 231/E1A 中轉染表現 IKKβ 的載體進去可表現磷酸化 FOXO3a 在 serine 644 的位置，且和 FOXO3a 對於 βTrCP 表現有關聯性（Figure 4F）。數據顯示出 E1A 調控 FOXO3a 磷酸化在 Serine 644 的位置是經由 TNF-α/IKKβ 所調控，並且會和 βTrCP 結合使其失去能力。

英文摘要

SUMMARY

Adenovirus type 5 E1A (E1A) induces sensitization to anticancer drug–induced apoptosis in different human cancers, including paclitaxel in breast and ovarian cancers. We found that Forkhead box O-class (FOXO) transcription factor FOXO3a is critical for E1A-mediated chemosensitization to paclitaxel. Knocked down FOXO3a expression dramatically abolished E1A-induced chemosensitization of paclitaxel. E1A stabilized FOXO3a by preventing ubiquitin-dependent proteolysis. The E3 ligase involved in stabilizing FOXO3a is β-transducin repeat–containing proteins (βTrCP); βTrCP binding to FOXO3a requires phosphorylation of FOXO3a at Ser644 by IKKβ. E1A reduces βTrCP-mediated ubiquitination of FOXO3a by inhibiting IKKβ activity. Further, inhibited IKKβ activity is due to E1A-induced expression of PP2A, which binds to transforming growth factor β–activated kinase 1 (TAK1), thus inhibiting TAK1's activation of IKKβ.

SIGNIFICANCE

E1A gene therapy was tested in multiple clinical trials in breast, ovarian, and head and neck cancers. E1A can sensitize paclitaxel-induced cell death and combined paclitaxel chemotherapy and E1A gene therapy is currently tested in a clinical trial for ovarian cancer patients. It has been shown that resistance to paclitaxel occurs in cells expressing low level of FOXO3a. In this study, we found that E1A stabilizes FOXO3a which is required to sensitize paclitaxel-induced apoptosis. The stabilization is achieved by E1A-induced expression of PP2A/C, which

inhibits the binding of TAK1 to IKKβ, therefore abolishing IKKβ's function in phosphorylating FOXO3a and FOXO3a degradation. This result suggests that the combination of *E1A* gene therapy and paclitaxel chemotherapy might be an effective way to treat paclitaxel-resistant tumors.

RESULTS

FOXO3a Is Critical for E1A-Mediated Chemosensitization

E1A gene therapy has been shown to induce chemosensitizations among different chemotherapeutic agents, including paclitaxel in breast and ovarian cancers (Liao et al., 2007). And it has been shown that resistance to paclitaxel occurs in cells expressing low level of FOXO3a (Sunters et al., 2003). We therefore ask whether FOXO3a may contribute to E1A-mediated chemosensitization. To this end, we examined the effects of E1A on FOXO3a expression in various types of cancer cells and NIH3T3 fibroblasts and found that expression of FOXO3a was significantly increased in *E1A*-transfected cells (Figure 1A). More importantly, E1A-induced chemosensitization of paclitaxel was abolished by knocked down FOXO3a expression using FOXO3a specific small interfering RNA (siFOXO3a) in MDA-MB-231 breast cancer cells as well as in SKOV3-ip1 ovarian cancer cells (Figure 1B). The expression of FOXO3a in siFOXO3a stable transfectants were also analyzed by Western bolt assay (Figure 1C). We further investigated the effects of FOXO3a on E1A-mediated chemosensitization in a xenograft tumor model in which mice were injected orthotopically with stably transfected cell clones. The results indicated that E1A induces the chemosensitization of paclitaxel *in vivo*, in that the tumor volume in 231/E1A-bearing mice treated with paclitaxel was significantly less than that in 231/vector-bearing mice

treated with paclitaxel (810.7 ± 73.2 mm^3 versus 1648.7 ± 237.4 mm^3; Figure 1D, lane 6 versus lane 2). More importantly, E1A-induced chemosensitization to paclitaxel was abolished by knocked down FOXO3a expression by stable expressing siFOXO3a in 231/E1A cells (810.7 ± 73.2 mm^3 versus 1855.1 ± 135.8 mm^3; Figure 1D, lane 6 versus lane 8). Increased tumor volumes by siFOXO3a treatment in 231/E1A correlated well with reduced FOXO3a expression in the tumors (Figure 1E). We therefore concluded that FOXO3a is required for the E1A-mediated chemosensitization to paclitaxel.

E1A Prevents Ub-Dependent Proteolysis of FOXO3a

Posttranslational modification and regulation of FOXO3a protein stability are critical for FOXO3a activity (Hu et al., 2004). Therefore, we attempted to determine the stability of FOXO3a protein in response to E1A in breast cancer cells. For this analysis, we treated control vector and E1A expression vector stable transfectants (231/vector and 231/E1A) with cycloheximide for various times to block de novo protein synthesis and found that the half-life of FOXO3a protein was more than 7 hours for E1A-transfected cells but less than 1.5 hours for control cells by western blot analysis (Figure 2A). TNFα-mediated FOXO3a polyubiquitination (Hu et al., 2004) was significantly decreased in 231/E1A cells compared with that in 231/vector cells (Figure 2B). These results suggest that E1A increases FOXO3a protein expression by preventing Ub-dependent proteolysis of FOXO3a.

βTrCP Is Involved in E1A-Induced FOXO3a Induction

In an attempt to search for the specific Ub E3 ligase responsible for

TNFα-induced FOXO3a polyubiquitination, we noticed that TNFα-activated IKK phosphorylates FOXO3a at Serine 644 (Hu et al., 2004) and the sequence surround Ser644 share a story homology to the consensus recognition site for βTrCP. Thus, we asked whether βTrCP was involved in FOXO3a protein degradation. To this end, we first asked whether βTrCP physically interacted with FOXO3a. We analyzed proteosome inhibitor MG132-treated 231/vector and 231/E1A cell lysates by reciprocal co-immunoprecipitation (IP) followed by immunoblotting (IB) using antibodies against FOXO3a and βTrCP. Our results showed that endogenous FOXO3a was associated with endogenous βTrCP in vivo in 231/vector cells and this interaction was stimulated by TNFα treatment. Interestingly, TNFα-induced binding between FOXO3a and βTrCP was significantly reduced in E1A-expressing cells (Figure 3A). This phenomenon was further confirmed by cotransfection of HA-FOXO3a with Myc-βTrCP into HeLa cells and analyzed by anti-HA and anti-Myc antibodies (Figure 3B). The F-box domain of βTrCP provides specificity by directly recruiting the substrate to the rest of the ligase and, ultimately, to the Ub-conjugating enzyme. In support of these notions, deletion of the F-box domain of βTrCP (Myc-βTrCP△F) cannot physically interact with FOXO3a (Figure 3C). In addition, βTrCP was shown to be required for FOXO3a expression by using siRNA of βTrCP. Cotransfection with siβTrCP1 and siβTrCP2 increased FOXO3a expression more dramatically than did transfection with either one alone in 231 vector cells, (Figure 3D, lane 4 versus lanes 2 and 3). Also, knockdown of βTrCP abolished the association between FOXO3a and βTrCP (Figure 3E). However, in E1A-expressing cells FOXO3a lost its ability to

interact with βTrCP (Figure 3D). Taken together, these results suggest that both βTrCP1 and βTrCP2 are involved in FOXO3a degradation. E1A inhibits interaction of FOXO3a and βTrCP which may prevent from FOXO3a degradation.

E1A Inhibits IKKβ-Mediated FOXO3a Phosphorylation at Ser644 and Prevents βTrCP-Induced FOXO3a Degradation

It is known that βTrCP preferentially recognizes phosphorylated Serine/Threonine in the consensus recognition site, and TNFα-activated IKK phosphorylates FOXO3a at Serine 644 (Hu et al., 2004). In supporting of this notion, the interaction of FOXO3a with βTrCP *in vivo* was shown to be enhanced by overexpression of IKKβ in HeLa cells (Figure 4A). In addition, the GFP-FOXO3a/S644E, a phosphorylation mimic mutant, interacts with βTrCP much stronger than that with GFP-FOXO3a/wild type (wt) (Figure 4B). FOXO3a is also known to be degraded by Akt through phosphorylation at 3 different sites (Thr32, Ser253, and Ser315) (Brunet et al., 1999). However, these Akt phosphorylated sites were not shown to be critical for interaction with βTrCP (Figure 4B).

The notion that phosphorylation of FOXO3a at Ser644 is required for binding with βTrCP was confirmed by the fact that competition with the FOXO3a peptide containing the Ser644 (peptide 636-654), but not the scrambled peptide, abolished the binding between FOXO3a and βTrCP (Figure 4C). Next, to confirm whether IKKβ is required for the association between FOXO3a and βTrCP, we cotransfected HA-FOXO3a with Myc-βTrCP into WT and IKKβ-deficient ($IKK\beta^{-/-}$) mouse embryonic fibroblasts (MEF) and

examined whether their interactions can be interrupted by IKKβ knockout. Figure 4D shows that the interaction between FOXO3a and βTrCP was present in WT MEFs but not in IKKβ$^{-/-}$ MEFs. When the IKKβ was re-expressed in IKKβ$^{-/-}$ MEFs, the interaction between FOXO3a and βTrCP was rescued (Figure 4D). Together, these data suggested that IKKβ-mediated phosphorylation of FOXO3a at Ser644 is required for binding to βTrCP.

To define whether E1A-mediated FOXO3a stabilization prevents FOXO3a phosphorylation at Ser644 by IKKβ and subsequent recognition by βTrCP, we demonstrated the phosphorylation status of FOXO3a at Ser644 and the association between FOXO3a and βTrCP in 231/vector and 231/E1A cells. Notably, TNFα-mediated FOXO3a phosphorylation at Ser644 was significantly decreased in 231/E1A cells (Figure 4E). Moreover, the TNFα-mediated association between FOXO3a and βTrCP was also reduced in 231/E1A cells (Figure 4E). Transfection with the IKKβ expression vector reestablished the phosphorylation of FOXO3a at Ser644 and the association between FOXO3a and βTrCP in 231/E1A cells (Figure 4F). These data indicated that the E1A-inhibited S644 phosphorylation of FOXO3a by TNFα/IKK and accordingly FOXO3a lost its ability to interact with βTrCP.

貳、97年度計畫著作一覽表

Journal

序號	計畫產出名稱	產出型式	Impact factor	致謝對象
	無			

Patent

序號	計畫產出名稱
	無

Book

序號	計畫產出名稱
	無

Conference Paper

序號	計畫產出名稱
	無

Technical Report

序號	計畫產出名稱
	無

參、97年度計畫重要研究成果產出統計表

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

(係指執行97年度計畫之所有研究產出結果)

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〔註〕：

期刊論文：指在學術性期刊上刊登之文章，其本文部份一般包含引言、方法、結果、及討論，並且一定有參考文獻部份，未在學術性期刊上刊登之文章（研究報告等）與博士或碩士論文，則不包括在內。

研討會論文：指參加學術性會議所發表之論文，且尚未在學術性期刊上發表者。

專　　著：為對某項學術進行專門性探討之純學術性作品。

技術報告：指從事某項技術之創新、設計及製程等研究發展活動所獲致的技術性報告且未公開發表者。

技術移轉：指技術由某個單位被另一個單位所擁有的過程。我國目前之技術轉移包括下列三項：一、技術輸入。二、技術輸出。三、技術擴散。

技術輸入：藉僑外投資、與外國技術合作、投資國外高科技事業等方式取得先進之技術引進國內者。

技術輸出：指直接供應國外買主具生產能力之應用技術、設計、顧問服務及專利等。我國技術輸出方包括整廠輸出、對外投資、對外技術合作及顧問服務等四種。

技術擴散：指政府引導式的技術移轉方式，即由財團法人、國營事業或政府研究機構將其開發之技術擴散至民間企業之一種單向移轉（政府移轉民間）。

技術創新：指研究執行中產生的技術，且有詳實技術資料文件者。

技術服務：凡有關各項研究計畫之規劃與評審、技術督察與指導及專業技術服務事項等。

肆、97年度計畫重要研究成果

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

計畫之新發現、新發明或對學術界、產業界具衝擊性(impact)之研究成果，請依性質勾選下列項目。

☑1. 研發或改良國人重要疾病及癌症的早期診斷方式及治療技術

☐2. 發展新的臨床治療方式

☐3. 發展新生物製劑、篩檢試劑及新藥品

☑4. 瞭解常見疾病及癌症之分子遺傳機轉

☑5. 瞭解抗癌藥劑對癌細胞之作用機制

☐6. 提供有效的疾病預防策略

☐7. 利用生物統計與生物資訊研究，推動台灣生技醫藥研究，促進生物技術與基因體醫學之發展

☐8. 醫療保健政策相關研究

☐9. 瞭解環境毒理機制及重金屬對人體健康的影響

☐10. 研發適合臨床使用的人造器官及生醫材料

☐11. 縮短復健流程並增加復健效果的醫療輔助方式或器材之研究應用

☐12. 改進現有醫療器材的功能或增加檢驗影像的解析能力

☐13. 其他重要疾病或醫藥衛生問題研究

一、 計畫之新發現、新發明或對學術界、產業界具衝擊性（impact）之研究成果，請敘述其執行情形。

乳癌是目前世界上女性癌症病患中為數最多的癌症之一，因此針對乳癌發展出嶄新的方式用以診斷、預防以及治療有其迫切的需求，將基礎研究的成果應用到臨床上以增進癌症病人的療效則是其中一項達成此目標的重要方式。報導已知第五型腺病毒蛋白(type5 E1A)和許多抗腫瘤作用有關，目前於多種癌症如乳癌、卵巢癌及頭頸癌之病患進行臨床上第二期之基因治療試驗。因此，了解與 E1A 相關之抗腫瘤作用中詳細之分子機制是相當重要，為了達到此目的，我們將著重於 E1A 所參與之訊息傳遞作用及 E1A 對於腫瘤生長的抑制作用，我們已發現多種可能與 E1A 抗腫瘤活性相關之作用，其中 E1A 在不同種類的癌細胞中會增加癌細胞對於抗癌藥物的敏感度，例如: 在乳癌及卵巢癌癌細胞中 E1A 會增強紫杉醇所引起的細胞凋亡。而我們發現 Forkhead box O-class (FOXO)轉錄因子 FOXO3a 在其中扮演十分重要的角色，利用 RNA 干擾技術去抑制 FOXO3a 的表現則可以有效減弱 E1A 所調節癌細胞對於紫杉醇的藥物敏感度，其中 E1A 穩定 FOXO3a 的表現是經由抑制泛素降解系統: β-transducin repeat–containing proteins (βTrCP)是 E3 泛素連接酶與穩定 FOXO3a 的表現有相當關係，而當 FOXO3a 被 IKKβ 磷酸化在 Ser644 的位置之後，βTrCP 則會因此與 FOXO3a 結合，進而透過泛素降解系統將 FOXO3a 降解，而 E1A 則可以透過抑制 IKKβ 的活性去達到抑制 βTrCP 對於 FOXO3a 所調節的泛素降解路徑。更進一步，我們發現 E1A 抑制 IKKβ 的活性是透過 protein phosphatae 2A(PP2A)這個絲氨酸及蘇氨酸去磷酸酶來進行調控，因為 E1A 會引起 PP2A 的表現量增加，增加

的 PP2A 會與 transforming growth factor β–activated kinase 1 (TAK1)結合，因此降低 TAK1 活性進而抑制 TGF-β 訊息傳遞路徑，使得 IKKβ 的活性也因此被抑制。這樣的結果提供了一個合理的解釋對於之後臨床應用上去合併 E1A 基因治療與紫杉醇化學治療，同時也將在設計標靶治療上提供一道新的曙光。目前計畫執行第一年，已達成本年度預定之進度，進展順利。

二、 計畫對民眾具教育宣導之研究成果（此部份將為規劃對一般民眾教育或宣導研究成果之依據，請以淺顯易懂之文字簡述研究成果，內容以不超過 300 字為原則）

乳癌是目前世界上女性癌症病患中為數最多的癌症之一，因此針對乳癌發展出嶄新的方式用以診斷、預防以及治療有其迫切的需求。本計劃針對合併 E1A 基因治療及紫杉醇化學治療的可能性進行探討，發現 E1A 基因治療可增加紫杉醇對於癌症細胞的敏感度，使治療效果更加顯著。因此未來若以此方向發展應可提供癌症治療的新穎策略。

三、 簡述年度計畫成果之討論與結論，如有技術移轉、技術推廣或業界合作，請概述情形及成效

計畫成果之結論：

1. FOXO3a 對於 E1A 調控之化療藥物增敏作用極具影響
2. E1A 防止 FOXO3a 被蛋白酶分解
3. βTrCP 參與 E1A 誘導 FOXO3a 的表現
4. E1A 經由抑制 IKKβ 調節 FOXO3a 在絲胺酸 644 的位置磷酸化和防止 βTrCP 誘發 FOXO3a 降解

根據此研究成果可提供了一個合理的解釋對於之後臨床應用上去合併

E1A 基因治療與紫杉醇化學治療，同時也將在設計標靶治療上提供一些新的策略及思考方向。

四、 成效評估（技術面、經濟面、社會面、整合綜效）

在技術面，我們已建立了許多實用的研究方法來探討本計劃所預期之成果，並且已經建立了研究所需要之細胞株等實驗材料，以作為下年度研究計畫進行所需。另外於經濟及社會方面，本計劃之成果可提供臨床治療上思考一種新的方向有關於合併基因治療與化學治療之可能性，若此方式可行，既可增加癌症的療效，又可因同時進行療程而減少病患住院所花的時間及醫療成本。綜上所述，本計劃提供了一個合理的解釋對於之後臨床應用上去合併 E1A 基因治療與紫杉醇化學治療。

五、 下年度工作構想及重點之妥適性

下年度將繼續完成計畫中尚未更深入探討之議題，我們將會詳細闡明E1A如何調控FOXO3a 之分子機制。此外，亦進入另一主題，探討E1A對於腫瘤轉移之抑制作用之相關研究。完成以上的主要目標能夠幫助我們對於E1A 之抗腫瘤作用建立更加詳細的分子訊息傳遞機制，其中包括 E1A 如何誘發細胞凋亡以及 E1A 如何抑制 epithelial-mesenchymal transition (EMT)、發炎反應所誘發之腫瘤生成和轉移作用。

六、 檢討與展望

本年度計畫執行情況及成果均在預計的進度內完成，並且有得到超過預期的實驗進度。希望來年能繼續探討 E1A 與癌症轉移之間的關係並發現更深入的結果。

伍、97年度計畫所培訓之研究人員

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

種類				人數	備註
專任人員	1.	博士後研究人員	訓練中	0	
			已結訓	0	
	2.	碩士級研究人員	訓練中	0	
			已結訓	0	
	3.	學士級研究人員	訓練中	0	
			已結訓	0	
	4.	其他	訓練中	0	
			已結訓	0	
兼任人員	1.	博士班研究生	訓練中	0	
			已結訓	0	
	2.	碩士班研究生	訓練中	2	
			已結訓	0	
醫師			訓練中	0	
			已結訓	0	

特殊訓練課程（請於備註欄說明所訓練課程名稱）

陸、參與97年度計畫所有人力之職級分析

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

職級	所含職級類別	參與人次
第一級	研究員、教授、主治醫師	0人
第二級	副研究員、副教授、總醫師、助教授	1人
第三級	助理研究員、講師、住院醫師	0人
第四級	研究助理、助教、實習醫師	1人
第五級	技術人員	0人
第六級	支援人員	0人
合計		2人

〔註〕：

第一級：研究員、教授、主治醫師、簡任技正，若非以上職稱則相當於博士滿三年、碩士滿六年、或學士滿九年之研究經驗者。

第二級：副研究員、副教授、助研究員、助教授、總醫師、薦任技正，若非以上職稱則相當於博士、碩士滿三年、學士滿六年以上之研究經驗者。

第三級：助理研究員、講師、住院醫師、技士，若非以上職稱則相當於碩士、或學士滿三年以上之研究經驗者。

第四級：研究助理、助教、實習醫師，若非以上職稱則相當於學士、或專科滿三年以上之研究經驗者。

第五級：指目前在研究人員之監督下從事與研究發展有關之技術性工作，且具備下列資格之一者屬之：具初（國）中、高中（職）、大專以上畢業者，或專科畢業目前從事研究發展，經驗未滿三年者。

第六級：指在研究發展執行部門參與研究發展有關之事務性及雜項工作者，如人事、會計、秘書、事務人員及維修、機電人員等。

柒、參與97年度計畫所有人力之學歷分析

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

類別	學　　歷　　別	參與人次
1	博士	1 人
2	碩士	1 人
3	學士	0 人
4	專科	0 人
5	博士班研究生	0 人
6	碩士班研究生	2 人
7	其他	0 人
合計		4 人

捌、參與97年度計畫所有協同合作之研究室

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

機構	研究室名稱	研究室負責人
The University of Texas MD Anderson Cancer Center	The Department of Molecular and Cellular Oncology, Dr. Mien-Chie Hung's Lab	Dr. Mien-Chie Hung

玖、九十七年度計畫執行情形

註：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

一、請簡述原計畫書中，九十七年預計達成之研究內容

The adenoviral type 5 E1A (E1A) associates with multiple anti-cancer activities and has been tested in multiple clinical trials in a gene therapy setting for breast, ovarian and head and neck cancer patients. Thus, it is critical and timely to understand the detailed molecular mechanisms that associate with E1A-mediated anti-cancer properties. We have unraveled several novel signaling pathways that may contribute to anti-cancer activities of E1A. The goal of this Career Development Grant application is to understand the molecular mechanisms of the E1A-mediated anti-cancer activities. To reach the goal, we will focus on how E1A may interact with the novel signaling pathways and exert suppression effects on tumor progression and metastasis in breast cancer.

In the first year, we hypothesized to define the regulatory mechanisms of FOXO3a expression and the biological function in response to E1A in breast cancer cells. We have identified a novel signaling cascade involving four key components: PP2A phosphatase and two kinases – TAK1 and IKK. We have shown that a forkhead transcriptional factor, FOXO3a associates with tumor suppression activity in breast cancer cells. Through inactivation of Akt and IKKα/β kinases, E1A may upregulate FOXO3a to enhance its anti-cancer activity. Our group have previously shown that E1A can repress TNF-α- and radiation-induced IKK activity, which causes stabilization of IκBαand therefore, inactivation of NF-κB. More recently, we also found that IKKα/βinhibits FOXO3a transcriptional activity through phosphorylation of FOXO3a resulting in nuclear exclusion and degradation. Thus, it prompts us to investigate whether

E1A may activate FOXO3a activity through suppression of IKK activity. To this end, we have shown that IKKβ-mediated repression of FOXO3a activity is indeed reversed in the presence of E1A, supporting the notion that E1A inhibits IKK activity to activate FOXO3a. This mechanism will be further tested in the first year of this grant.

二、請詳述九十七年度計畫執行情形，並評估是否已達到原預期目標（請註明達成率）

Adenovirus type 5 E1A (E1A) induces sensitization to anticancer drug–induced apoptosis in different human cancers, including paclitaxel in breast and ovarian cancers. We found that Forkhead box O-class (FOXO) transcription factor FOXO3a is critical for E1A-mediated chemosensitization to paclitaxel. Knocked down FOXO3a expression dramatically abolished E1A-induced chemosensitization of paclitaxel. E1A stabilized FOXO3a by preventing ubiquitin-dependent proteolysis. The E3 ligase involved in stabilizing FOXO3a is β-transducin repeat–containing proteins (βTrCP); βTrCP binding to FOXO3a requires phosphorylation of FOXO3a at Ser644 by IKKβ. E1A reduces βTrCP-mediated ubiquitination of FOXO3a by inhibiting IKKβ activity. In the first year, we successfully set up our in vitro and in vivo system and get the anticipated results.

RESULTS

FOXO3a Is Critical for E1A-Mediated Chemosensitization

E1A gene therapy has been shown to induce chemosensitizations among different chemotherapeutic agents, including paclitaxel in breast and ovarian cancers (Liao et al., 2007). And it has been shown that resistance to paclitaxel occurs in cells expressing low level of FOXO3a (Sunters et al., 2003). We

therefore ask whether FOXO3a may contribute to E1A-mediated chemosensitization. To this end, we examined the effects of E1A on FOXO3a expression in various types of cancer cells and NIH3T3 fibroblasts and found that expression of FOXO3a was significantly increased in *E1A*-transfected cells (Figure 1A). More importantly, E1A-induced chemosensitization of paclitaxel was abolished by knocked down FOXO3a expression using FOXO3a specific small interfering RNA (siFOXO3a) in MDA-MB-231 breast cancer cells as well as in SKOV3-ip1 ovarian cancer cells (Figure 1B). The expression of FOXO3a in siFOXO3a stable transfectants were also analyzed by Western bolt assay (Figure 1C). We further investigated the effects of FOXO3a on E1A-mediated chemosensitization in a xenograft tumor model in which mice were injected orthotopically with stably transfected cell clones. The results indicated that E1A induces the chemosensitization of paclitaxel *in vivo*, in that the tumor volume in 231/E1A-bearing mice treated with paclitaxel was significantly less than that in 231/vector-bearing mice treated with paclitaxel (810.7 ± 73.2 mm^3 versus 1648.7 ± 237.4 mm^3; Figure 1D, lane 6 versus lane 2). More importantly, E1A-induced chemosensitization to paclitaxel was abolished by knocked down FOXO3a expression by stable expressing siFOXO3a in 231/E1A cells (810.7 ± 73.2 mm^3 versus 1855.1 ± 135.8 mm^3; Figure 1D, lane 6 versus lane 8). Increased tumor volumes by siFOXO3a treatment in 231/E1A correlated well with reduced FOXO3a expression in the tumors (Figure 1E). We therefore concluded that FOXO3a is required for the E1A-mediated chemosensitization to paclitaxel.

E1A Prevents Ub-Dependent Proteolysis of FOXO3a

Posttranslational modification and regulation of FOXO3a protein stability are critical for FOXO3a activity (Hu et al., 2004). Therefore, we attempted to determine the stability of FOXO3a protein in response to E1A in breast cancer

cells. For this analysis, we treated control vector and E1A expression vector stable transfectants (231/vector and 231/E1A) with cycloheximide for various times to block de novo protein synthesis and found that the half-life of FOXO3a protein was more than 7 hours for E1A-transfected cells but less than 1.5 hours for control cells by western blot analysis (Figure 2A). TNFα-mediated FOXO3a polyubiquitination (Hu et al., 2004) was significantly decreased in 231/E1A cells compared with that in 231/vector cells (Figure 2B). These results suggest that E1A increases FOXO3a protein expression by preventing Ub-dependent proteolysis of FOXO3a.

βTrCP Is Involved in E1A-Induced FOXO3a Induction

In an attempt to search for the specific Ub E3 ligase responsible for TNFα-induced FOXO3a polyubiquitination, we noticed that TNFα-activated IKK phosphorylates FOXO3a at Serine 644 (Hu et al., 2004) and the sequence surround Ser644 share a story homology to the consensus recognition site for βTrCP. Thus, we asked whether βTrCP was involved in FOXO3a protein degradation. To this end, we first asked whether βTrCP physically interacted with FOXO3a. We analyzed proteosome inhibitor MG132-treated 231/vector and 231/E1A cell lysates by reciprocal co-immunoprecipitation (IP) followed by immunoblotting (IB) using antibodies against FOXO3a and βTrCP. Our results showed that endogenous FOXO3a was associated with endogenous βTrCP in vivo in 231/vector cells and this interaction was stimulated by TNFα treatment. Interestingly, TNFα-induced binding between FOXO3a and βTrCP was significantly reduced in E1A-expressing cells (Figure 3A). This phenomenon was further confirmed by cotransfection of HA-FOXO3a with Myc-βTrCP into HeLa cells and analyzed by anti-HA and anti-Myc antibodies (Figure 3B). The F-box domain of βTrCP provides specificity by directly recruiting the substrate to the rest of the ligase and, ultimately, to the Ub-conjugating enzyme. In

support of these notions, deletion of the F-box domain of βTrCP (Myc-βTrCP△F) cannot physically interact with FOXO3a (Figure 3C). In addition, βTrCP was shown to be required for FOXO3a expression by using siRNA of βTrCP. Cotransfection with siβTrCP1 and siβTrCP2 increased FOXO3a expression more dramatically than did transfection with either one alone in 231 vector cells, (Figure 3D, lane 4 versus lanes 2 and 3). Also, knockdown of βTrCP abolished the association between FOXO3a and βTrCP (Figure 3E). However, in E1A-expressing cells FOXO3a lost its ability to interact with βTrCP (Figure 3D). Taken together, these results suggest that both βTrCP1 and βTrCP2 are involved in FOXO3a degradation. E1A inhibits interaction of FOXO3a and βTrCP which may prevent from FOXO3a degradation.

E1A Inhibits IKKβ-Mediated FOXO3a Phosphorylation at Ser644 and Prevents βTrCP-Induced FOXO3a Degradation

It is known that βTrCP preferentially recognizes phosphorylated Serine/Threonine in the consensus recognition site, and TNFα-activated IKK phosphorylates FOXO3a at Serine 644 (Hu et al., 2004). In supporting of this notion, the interaction of FOXO3a with βTrCP *in vivo* was shown to be enhanced by overexpression of IKKβ in HeLa cells (Figure 4A). In addition, the GFP-FOXO3a/S644E, a phosphorylation mimic mutant, interacts with βTrCP much stronger than that with GFP-FOXO3a/wild type (wt) (Figure 4B). FOXO3a is also known to be degraded by Akt through phosphorylation at 3 different sites (Thr32, Ser253, and Ser315) (Brunet et al., 1999). However, these Akt phosphorylated sites were not shown to be critical for interaction with βTrCP (Figure 4B).

The notion that phosphorylation of FOXO3a at Ser644 is required for binding with βTrCP was confirmed by the fact that competition with the

FOXO3a peptide containing the Ser644 (peptide 636-654), but not the scrambled peptide, abolished the binding between FOXO3a and βTrCP (Figure 4C). Next, to confirm whether IKKβ is required for the association between FOXO3a and βTrCP, we cotransfected HA-FOXO3a with Myc-βTrCP into WT and IKKβ-deficient ($IKK\beta^{-/-}$) mouse embryonic fibroblasts (MEF) and examined whether their interactions can be interrupted by IKKβ knockout. Figure 4D shows that the interaction between FOXO3a and βTrCP was present in WT MEFs but not in $IKK\beta^{-/-}$ MEFs. When the IKKβ was re-expressed in $IKK\beta^{-/-}$ MEFs, the interaction between FOXO3a and βTrCP was rescued (Figure 4D). Together, these data suggested that IKKβ-mediated phosphorylation of FOXO3a at Ser644 is required for binding to βTrCP.

To define whether E1A-mediated FOXO3a stabilization prevents FOXO3a phosphorylation at Ser644 by IKKβ and subsequent recognition by βTrCP, we demonstrated the phosphorylation status of FOXO3a at Ser644 and the association between FOXO3a and βTrCP in 231/vector and 231/E1A cells. Notably, TNFα-mediated FOXO3a phosphorylation at Ser644 was significantly decreased in 231/E1A cells (Figure 4E). Moreover, the TNFα-mediated association between FOXO3a and βTrCP was also reduced in 231/E1A cells (Figure 4E). Transfection with the IKKβ expression vector reestablished the phosphorylation of FOXO3a at Ser644 and the association between FOXO3a and βTrCP in 231/E1A cells (Figure 4F). These data indicated that the E1A-inhibited S644 phosphorylation of FOXO3a by TNFα/IKK and accordingly FOXO3a lost its ability to interact with βTrCP.

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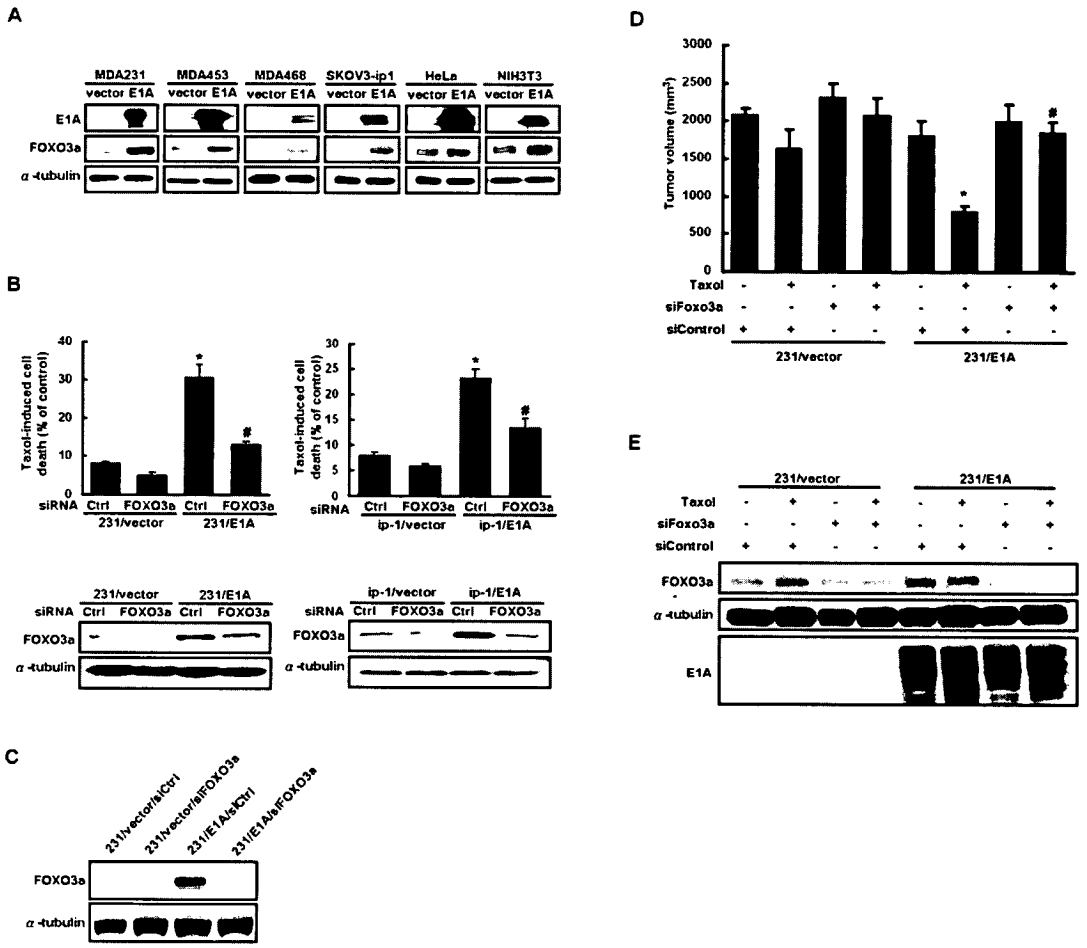
Figure 1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B
Taxol-induced cell death (% of control)
siRNA
Ctrl
FOXO3a
231/vector
231/E1A
ip-1/vector
ip-1/E1A
C
231/vector/siCtrl
231/vector/siFOXO3a
231/E1A/siCtrl
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Tumor volume (mm³)
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siFoxo3a
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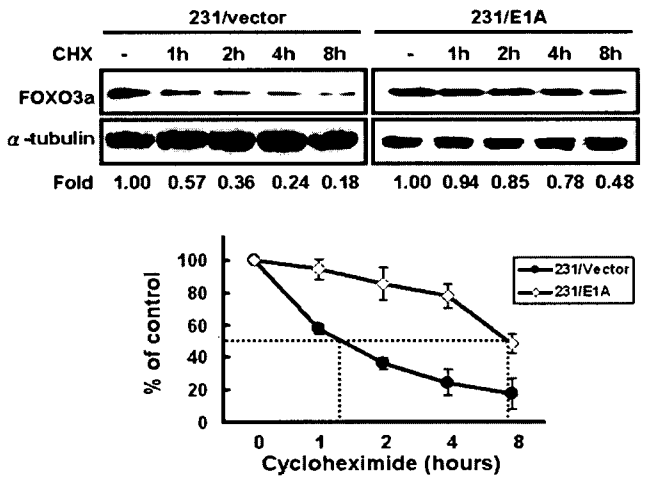
E
E1A

Figure 2

A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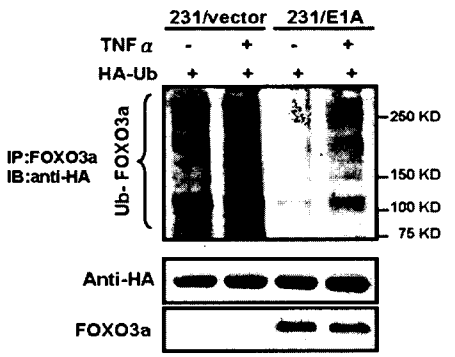
231/Vector
231/E1A

B


231/vector 231/E1A
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HA-Ub + + + +
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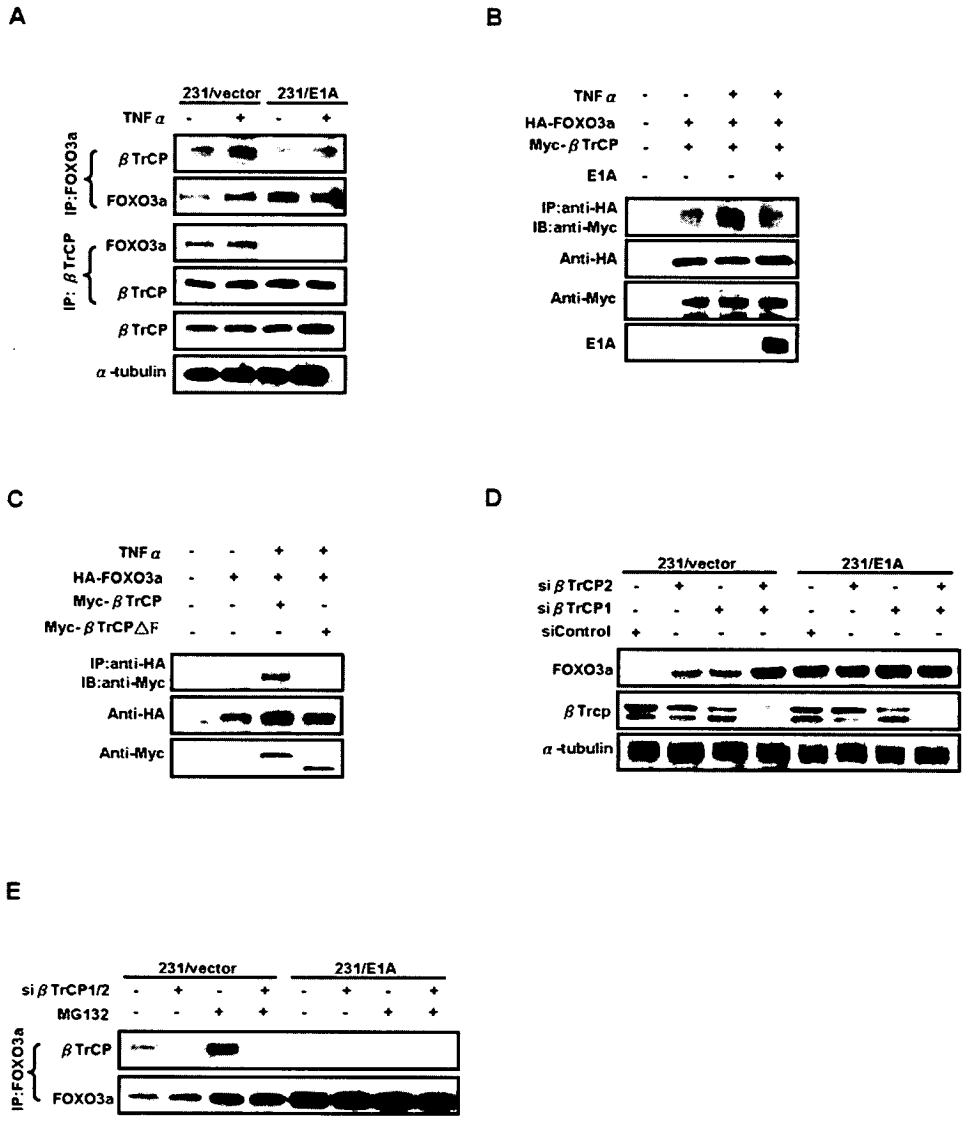
100 KD
75 KD
Anti-HA
FOXO3a

Figure 3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231/vector
231/E1A
TNF α
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βTrCP
FOXO3a
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B
TNF α
HA-FOXO3a
Myc-βTrCP
E1A
IP:anti-HA
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C
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Myc-βTrCPΔF
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IB:anti-Myc
Anti-HA
Anti-Myc
D
231/vector
231/E1A
siβTrCP2
siβTrCP1
siControl
FOXO3a
βTrcp
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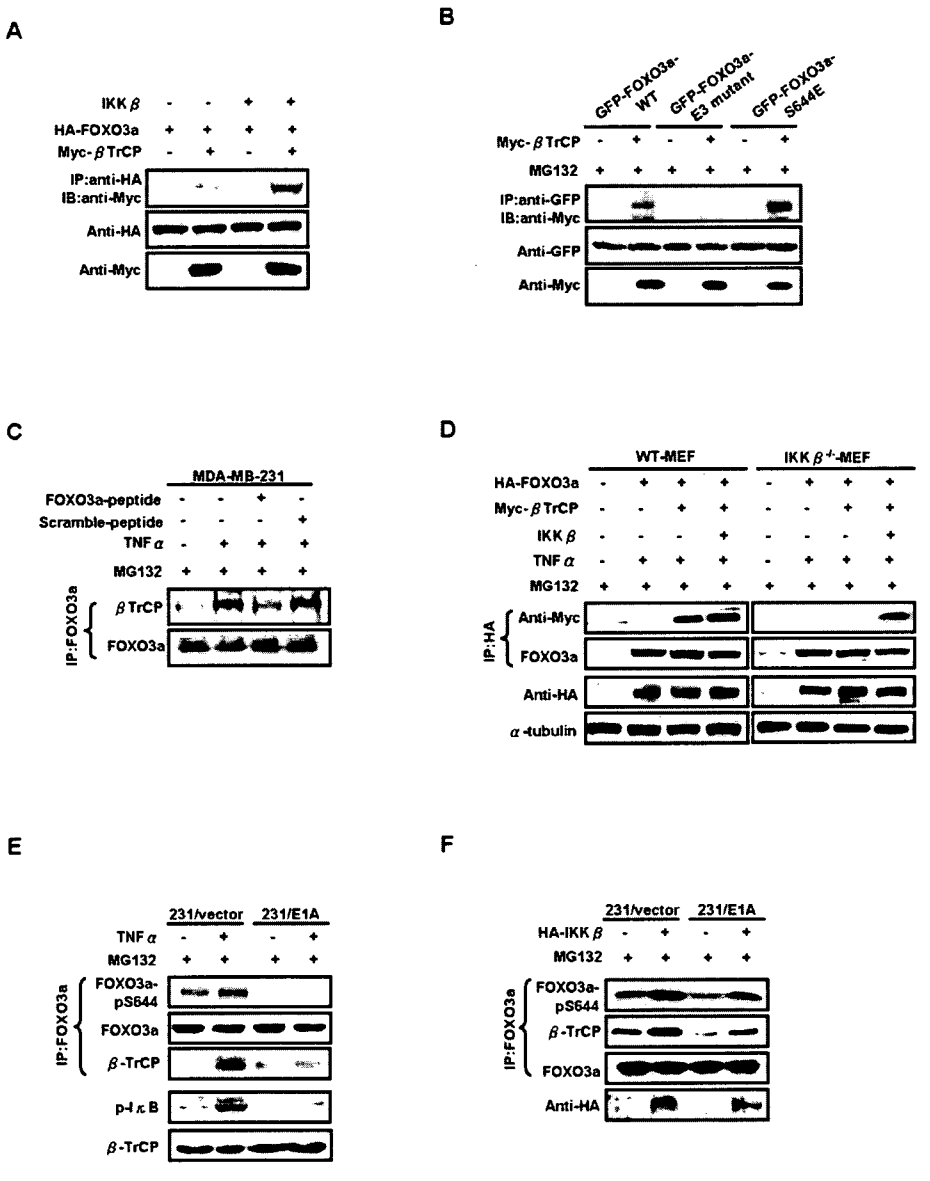
Figure 4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B
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GFP-FOXO3a-S644E
Myc-βTrCP
MG132
IP:anti-GFP
IB:anti-Myc
Anti-GFP
Anti-Myc
C
MDA-MB-231
FOXO3a-peptide
Scramble-peptide
TNFα
MG132
IP:FOXO3a
βTrCP
FOXO3a
D
WT-MEF
IKKβ-/--MEF
HA-FOXO3a
Myc-βTrCP
IKKβ
TNFα
MG132
IP:HA
Anti-Myc
FOXO3a
Anti-HA
α-tubulin
E
231/vector
231/E1A
TNFα
MG132
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MG132
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β-TrCP
FOXO3a
Anti-HA

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E1A-Induced PP2A Expression Prevents βTrCP-Mediated FOXO3a Degradation and Enhances Chemosensitization by Inhibiting TAK1/IKK Signaling

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Running title: FOXO3a Is Involved in Chemosensitization

SUMMARY

Adenovirus type 5 E1A (E1A) induces sensitization to anticancer drug–induced apoptosis in different human cancers, including paclitaxel in breast and ovarian cancers. We found that Forkhead box O-class (FOXO) transcription factor FOXO3a is critical for E1A-mediated chemosensitization to paclitaxel. Knocked down FOXO3a expression dramatically abolished E1A-induced chemosensitization of paclitaxel. E1A stabilized FOXO3a by preventing ubiquitin-dependent proteolysis. The E3 ligase involved in stabilizing FOXO3a is β-transducin repeat–containing proteins (βTrCP); βTrCP binding to FOXO3a requires phosphorylation of FOXO3a at Ser644 by IKKβ. E1A reduces βTrCP-mediated ubiquitination of FOXO3a by inhibiting IKKβ activity. Further, inhibited IKKβ activity is due to E1A-induced expression of PP2A, which binds to transforming growth factor β–activated kinase 1 (TAK1), thus inhibiting TAK1's activation of IKKβ.

SIGNIFICANCE

E1A gene therapy was shown safe and well tolerated by multiple clinical trials in breast, ovarian, or head and neck cancers. Additionally, it might be an effective strategy for enhancing the sensitivity of tumor cells to chemotherapy. Paclitaxel is a chemotherapeutic agent for breast and ovarian cancer. Resistance to paclitaxel occurs in cells expressing low level of FOXO3a. In this study, we found that E1A stabilizes FOXO3a and sensitizes paclitaxel-induced apoptosis. The stabilization is achieved by E1A-induced expression of PP2A/C, which inhibits the binding of TAK1 to IKKβ, therefore abolishing IKKβ's function in phosphorylating FOXO3a and FOXO3a degradation. This result suggests that the combination of *E1A* gene therapy and paclitaxel chemotherapy might be an effective way to treat paclitaxel-resistant tumors.

INTRODUCTION

Adenovirus type 5 E1A (E1A) was originally recognized as an oncogene that could facilitate oncogenic transformation by other viral and cellular oncogenes. However, E1A has not been associated with human malignancies despite extensive efforts to identify such a link (Yu and Hung, 1998). Instead, E1A was shown to associate with antitumor activities by reversing the transformed phenotype, inhibiting metastasis, and inducing apoptosis in multiple transformed rodent cells and human cancer cell lines (Deng et al., 1998; Ueno et al., 2001; Yu et al., 1992). In addition to the tumor suppressor activities, expression of the *E1A* gene in stably transfected normal fibroblasts and human cancer cells has also been shown to induce chemosensitization among different categories of anticancer drugs in vitro, including etoposide in normal fibroblasts, rhabdomyosarcoma, osteosarcoma, non-small cell lung cancer and breast cancer cells (Frisch and Dolter, 1995; Lowe et al., 1993; Zhou et al., 2001); cisplatin in keratinocytes, sarcoma, breast, and ovarian cancer cells (Brader et al., 1997; Frisch and Dolter, 1995; Liao and Hung, 2003; Sanchez-Prieto et al., 1995a; Sanchez-Prieto et al., 1995b; Viniegra et al., 2002); doxorubicin in normal fibroblasts, sarcoma, and breast cancer cells (Liao and Hung, 2003; Lowe et al., 1993; Zhou et al., 2001), gemcitabine in hepatocellular and breast cancer cells (Lee et al., 2003; Liao and Hung, 2003); Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in ovarian cancer cells (Shao et al., 2005); and paclitaxel in breast and ovarian cancers (Brader et al., 1997; Liao and Hung, 2003; Ueno et al., 2000; Ueno et al., 1997). Animal studies also showed that the combination of systemic *E1A* gene therapy with paclitaxel significantly enhanced paclitaxel-induced apoptosis and prolonged survival rates in the animal orthotopic model in vivo (Liao et al., 2004). Therefore, *E1A* is now considered a tumor suppressor gene and has been tested in multiple clinical trials in a gene therapy setting for patients with breast (Hortobagyi et al., 1998;

Hortobagyi et al., 2001; Yoo et al., 2001), ovarian (Hortobagyi et al., 1998; Hortobagyi et al., 2001; Madhusudan et al., 2004), or head and neck cancers (Villaret et al., 2002; Yoo et al., 2001). This evidence suggests that *E1A* gene therapy might be an effective strategy for enhancing the sensitivity of tumor cells to chemotherapy. However, the molecular mechanisms underlying E1A-mediated chemosensitization are still not completely defined. It is critical and timely to understand the detailed molecular mechanisms that associate with E1A-mediated chemosensitization so future clinical trials using the combination of chemotherapy with *E1A* gene therapy can be developed.

One of the mechanisms by which E1A induces chemosensitization is down-regulation of Her-2/neu overexpression (Brader et al., 1997; Ueno et al., 2000; Yu and Hung, 2000a; Yu and Hung, 2000b; Zhou et al., 2001). Recently, down-regulation of Akt activation of p38 was reported to provide a general cellular mechanism for E1A-mediated chemosensitization (Liao and Hung, 2003; Liao and Hung, 2004). Regulation of some critical tumor suppressors was also proposed as being involved in E1A-induced chemosensitization, such as p53 and p19ARF (de Stanchina et al., 1998; Lowe, 1999; Lowe et al., 1993), the proapoptotic protein Bax, caspase 9, and a yet-unidentified inhibitor that ordinarily provides protection against cell death (Duelli and Lazebnik, 2000; Fearnhead et al., 1998; McCurrach et al., 1997; Putzer et al., 2000; Teodoro et al., 1995; Ueno et al., 2000).

Forkhead box O–class (FOXO) transcription factors include FOXO1 (Forkhead in rhabdomyosarcoma, FKHR), FOXO3a (FKHR-like 1, FKHRL1), and FOXO4 (acute lymphocytic leukemia–fused gene from chromosome X, AFX). The FOXOs activate and/or repress transcription of genes involved in metabolism, apoptosis, DNA damage repair, and cell cycle progression (Accili and Arden, 2004). For example, FOXO3a activity has been shown to

elevate $p27^{kip}$ expression and induce cell cycle arrest (Medema et al., 2000). FOXO3a and FOXO4 have also been shown to inhibit the cell cycle through down-regulation of cyclin D by a $p27^{kip}$-independent mechanism (Dijkers et al., 2000b; Schmidt et al., 2002). In breast cancer, FOXO3a has been shown to up-regulate BIM, a BH3 domain protein very effective at inducing apoptosis (Dijkers et al., 2000a; Gilley et al., 2003). The activity of the FOXOs can be inhibited by activating the phosphoinositide 3'-kinase (PI3K)/Akt pathway. FOXO3a can be phosphorylated by Akt at three conserved serine/threonine residues (Thr32, Ser253, and Ser315), and it subsequently translocates from the nucleus to the cytoplasm, where it is retained by binding to the 14-3-3 protein (Brunet et al., 1999). FOXO3a activity can also be inhibited by IκB kinase (IKK) signaling pathway. IKK physically interacts with and phosphorylates FOXO3a independently of Akt, which causes nuclear exclusion of FOXO3a and subsequently proteolysis of FOXO3a via the ubiquitin (Ub)-dependent proteasome pathway (Hu et al., 2004). However, the contributing E3 Ub ligase and detailed molecular mechanism of FOXO3a proteolysis are still unclear.

In an attempt to understand the molecular mechanism of E1A-mediated chemosensitization, we found that FOXO3a is critical to that process. E1A stabilizes FOXO3a by preventing Ub-dependent proteolysis mediated by E3 ligase β-transducin repeat–containing proteins (βTrCP). The binding of βTrCP to FOXO3a requires the phosphorylation of FOXO3a at Ser644 by IKKβ. E1A induces the expression of PP2A (a protein phosphatase involved in multiple cellular functions, including chemosensitization), which is involved in inhibiting transforming growth factor β–activated kinase 1 (TAK1)-activated IKK signaling, therefore stabilizing FOXO3a and inducing chemosensitization.

RESULTS

FOXO3a Is Critical for E1A-Mediated Chemosensitization

To examine the effects of E1A on FOXO3a expression, various types of cancer cells and NIH3T3 fibroblasts were transfected with *E1A* expression vector. Expression of FOXO3a was significantly increased in *E1A*-transfected cells (Figure 1A). More importantly, E1A-induced chemosensitization of paclitaxel was dramatically abolished by knocked down FOXO3a expression using FOXO3a specific small interfering RNA (siFOXO3a) in MDA-MB-231 breast cancer cells as well as in SKOV3-ip1 ovarian cancer cells (Figure 1B). We further investigated the effects of FOXO3a on E1A-mediated chemosensitization in a xenograft tumor model in which mice were injected orthotopically with stably transfected cell clones. After 6 weeks, the tumors in mice injected with MDA-MB-231/vector cells were slightly larger than those in mice injected with MDA-MB-231/E1A clones (231/vector, mean ± SE = 2097.4 ± 72.6 mm^3; 231/E1A, mean ± SE = 1815.0 ± 190.5 mm^3; Figure 1C, lane 1 versus lane 4). Furthermore, the tumor volume in 231/E1A-bearing mice treated with paclitaxel was significantly less than that in 231/vector-bearing mice treated with paclitaxel (810.7 ± 73.2 mm^3 versus 1648.7 ± 237.4 mm^3; Figure 1C, lane 6 versus lane 2). More importantly, E1A-induced chemosensitization to paclitaxel was dramatically abolished by knocked down FOXO3a expression by stable expressing siFOXO3a in 231/E1A cells (810.7 ± 73.2 mm^3 versus 1855.1 ± 135.8 mm^3; Figure 1C, lane 6 versus lane 8). In addition, E1A-mediated FOXO3a expression in tumors was significantly inhibited by siFOXO3a (Figure 1D). We therefore concluded that FOXO3a plays a critical role in E1A-mediated chemosensitization to paclitaxel.

E1A Prevents Ub-Dependent Proteolysis of FOXO3a

Posttranslational modification and regulation of FOXO3a protein stability are critical for FOXO3a activity (Hu et al., 2004). Therefore, we attempted to determine the stability of FOXO3a protein in response to E1A in breast cancer cells. For this analysis, we treated control vector and E1A expression vector stable transfectants (231/vector and 231/E1A) with cycloheximide for various times to block de novo protein synthesis. According to Western blot analysis and the resultant quantitative data, the half-life of FOXO3a protein was more than 7 hours for E1A-transfected cells but less than 1.5 hours for control cells (Figure 2A). Furthermore, we demonstrated polyubiquitination of FOXO3a in 231/vector cells in response to tumor necrosis factor α (TNFα) treatment, which has been reported to induce polyubiquitination of FOXO3a (Hu et al., 2004). Notably, TNFα-mediated FOXO3a ubiquitination was significantly decreased in 231/E1A cells (Figure 2B). These results suggest that E1A increases FOXO3a protein expression by preventing Ub-dependent proteolysis of FOXO3a.

βTrCP Is Involved in E1A-Induced FOXO3a Induction

The proteolysis of FOXO3a reportedly occurs via the Ub-dependent proteosome pathway (Hu et al., 2004), but which Ub E3 ligase is involved in this degradation process was unknown. Because βTrCP Ub ligase is responsible for the degradation of multiple proteins by recruiting the ubiquitination machinery to its substrates and mediating the conjugation of multiple Ub's (Cardozo and Pagano, 2004), we asked whether βTrCP was also involved in FOXO3a protein degradation. To define whether βTrCP physically interacts with FOXO3a, we analyzed proteosome inhibitor MG132-treated 231/vector and 231/E1A cell lysates by reciprocal co-immunoprecipitation (IP) followed by immunoblotting (IB) using antibodies against FOXO3a and βTrCP. Our results showed that endogenous FOXO3a was specifically associated with

endogenous βTrCP in vivo in 231/vector cells but not in 231/E1A cells (Figure 3A). We found that TNFα-induced binding between endogenous FOXO3a and βTrCP appeared to be abolished in E1A-expressing cells.

To confirm this association with tag-specific antibody, we cotransfected HA-FOXO3a with Myc-βTrCP into HeLa cells and examined whether their interaction could be interrupted by E1A expression. After IP/IB with anti-HA and anti-Myc antibodies, the exogenously expressed HA-FOXO3a specifically interacted with Myc-βTrCP in vivo, and this interaction was abolished by expression of E1A (Figure 3B). The F-box protein subunit of βTrCP provides specificity by directly recruiting the substrate to the rest of the ligase and, ultimately, to the Ub-conjugating enzyme. In support of these results, deletion of the F-box protein subunit, of βTrCP (Myc-βTrCP △F) cannot physically interact with FOXO3a (Figure 3C). Because the sequence surrounding Ser644 (the site of FOXO3a phosphorylation by IKK) shows a strong homology with the consensus recognition site for βTrCP, IKK activity is expected to be required for recruiting βTrCP to FOXO3a. Indeed, the interaction of FOXO3a with βTrCP *in vivo* was significantly enhanced by overexpression of IKKβ in HeLa cells (Figure 3D). These data indicated that FOXO3a is physically associated with βTrCP and that this interaction may require the involvement of TNFα- and IKKβ-mediated signaling.

Given this physical association, we next determined whether βTrCP is required for FOXO3a processing by using small interfering RNA (siRNA) to knock down the endogenous *βTrCP* gene product. Transfection of double-stranded βTrCP1 or βTrCP2 siRNA, which was previously shown to effectively knock down βTrCP1 or βTrCP2 in human breast cancer cells (Tang et al., 2005), efficiently down-regulated the production of endogenous βTrCP species and increasee the FOXO3a protein level (Figure 3E). Cotransfection with siβTrCP1 and siβTrCP2

increased FOXO3a expression more dramatically than did transfection with either one alone (Figure 3E, lane 4 versus lanes 2 and 3). Consistent with our hypothesis, knockdown of βTrCP also abolished the association between FOXO3a and βTrCP (Figure 3F) and prevented βTrCP-mediated FOXO3a degradation (Figure 3E). These data indicated that βTrCP is the critical Ub E3 ligase involved in E1A-mediated FOXO3a degradation.

E1A Inhibits IKKβ-Mediated FOXO3a Phosphorylation at Ser644 and Prevents βTrCP-Induced FOXO3a Degradation

Most βTrCP substrates are recognized and bound by the F-box protein subunit only when they are phosphorylated on specific sites. To determine which phosphorylation pattern of FOXO3a is required for βTrCP interaction, all three Akt phosphorylation sites of FOXO3a (Thr32, Ser253, and Ser315) and the IKKβ phosphorylation site of FOXO3a (Ser644) were mutated to glutamine to mimic the phosphorylated status of FOXO3a. Green fluorescent protein (GFP)-tagged FOXO3a/3E mutant or GFP-FOXO3a/S644E mutant were cotransfected with Myc-βTrCP in HeLa cells. Our results showed that βTrCP has a preference for associating with GFP-FOXO3a/S644E rather than with GFP-FOXO3a/wild type (WT) and GFP-FOXO3a/3E (Figure 4A).

To confirm that phosphorylation of FOXO3a at Ser644 is required for binding with βTrCP, we mutated Ser644 into alanine (S644A) and found that the IKKβ-induced association between FOXO3a and βTrCP was abrogated (Figure 4B). Accordingly, competition with the FOXO3a peptide containing the Ser644 (peptide 636-654), but not the scrambled peptide, abolished the binding between FOXO3a and βTrCP (Figure 4C). Next, to confirm that IKKβ is required for the association between FOXO3a and βTrCP, we cotransfected HA-FOXO3a with Myc-βTrCP into WT and IKKβ-deficient ($IKK\beta^{-/-}$) mouse embryonic fibroblasts (MEF) and examined whether

their interactions can be interrupted by IKKβ knockout. Figure 4D shows that the interaction between FOXO3a and βTrCP was present in WT MEFs but not in IKKβ$^{-/-}$ MEFs. When the IKKβ-expressing vector was re-expressed in IKKβ$^{-/-}$ MEFs, the interaction between FOXO3a and βTrCP was rescued (Figure 4D). These data indicated that IKKβ-mediated phosphorylation of FOXO3a at Ser644 is required for binding to βTrCP.

To define whether E1A-mediated FOXO3a stabilization prevents FOXO3a phosphorylation at Ser644 by IKKβ and subsequent recognition by βTrCP, we demonstrated the phosphorylation status of FOXO3a at Ser644 and the association between FOXO3a and βTrCP in 231/vector and 231/E1A cells. Notably, TNFα-mediated FOXO3a phosphorylation at Ser644 was significantly decreased in 231/E1A cells (Figure 4E). Moreover, the TNFα-mediated association between FOXO3a and βTrCP was also reduced in 231/E1A cells (Figure 4E). Transfection with the IKKβ expression vector reestablished the phosphorylation of FOXO3a at Ser644 and the association between FOXO3a and βTrCP in 231/E1A cells (Figure 4F). These data indicated that the E1A-mediated prevention of FOXO3a phosphorylation and the interaction with βTrCP occurred through inhibition of IKKβ activity.

To further define whether IKKβ is required for E1A-induced chemosensitization, we transiently transfected E1A expression vector with or without IKKβ expression vector into MDA-MB-231 cells and determined the effects of paclitaxel-induced cell death. E1A-induced chemosensitization was strikingly abolished by transfection with IKKβ expression vector (Figure 4G). Additionally, treatment with two chemical inhibitors, BAY117082 and parthenolide, to inhibit IKK activity also increased the chemosensitization of MDA-MB-231 cells (Figure 4G) to paclitaxel. These results indicate that IKKβ activity is critical for chemosensitization of breast and ovarian cancer cells in both E1A-dependent and -independent manners.

Down-regulation of TAK1 Activity Is Critical for E1A-Mediated FOXO3a Stabilization and Chemosensitization

To explore the mechanism(s) through which inhibition of IKK activity participates in the cellular responses to E1A, we determined the phosphorylation of IKK in 231/vector and 231/E1A cells. Treatment with TNFα increased the phosphorylation of IKK in 231/vector cells, but this activation was abolished in 231/E1A cells (Figure 5A). These data indicated that E1A-induced inhibition of IKK signaling may target the upstream kinase of IKK. Recent evidence indicates that TAK1 is essential for the activation of IKK in multiple signaling pathways (Sato et al., 2005). Therefore, the involvement of TAK1 in E1A-mediated inhibition of IKK signaling, FOXO3a stabilization, and chemosensitization were of interest. We found that treatment with TNFα increased the phosphorylation of TAK1 in 231/vector cells, and this phosphorylation was diminished by expression of E1A (Figure 5B). Furthermore, transfection with the HA-TAK1 expression vector significantly increased phosphorylation of IKK and subsequently degradation of FOXO3a in 231/E1A cells (Figure 5C).

Experiments were also performed to ascertain whether TAK1 is involved in E1A-mediated FOXO3a phosphorylation at Ser644 and chemosensitization. Overexpression of HA-TAK1 significantly increased FOXO3a phosphorylation at Ser644 and the interaction between FOXO3a and βTrCP in both 231/vector and 231/E1A cells (Figure 5D). More importantly, E1A-mediated chemosensitization to paclitaxel was also significantly impaired by expression of TAK1 (Figure 5E). These data indicate that TAK1, the upstream kinase of IKK, is critical for E1A-mediated FOXO3a phosphorylation and subsequent chemosensitization.

E1A-Induced PP2A Expression Is Required for Regulation of IKK Signaling, FOXO3a Phosphorylation and Chemosensitization

Phosphorylation of protein kinases are tightly regulated by related protein phosphatases, and it has been reported that E1A increases the expression of PP2A (Liao and Hung, 2004). To identify whether PP2A was involved in E1A-mediated down-regulation of TAK1 and IKK activation and FOXO3a stabilization, 231/vector and 231/E1A cells were treated with the phosphatase inhibitor okadaic acid (OA). We found that E1A-mediated inhibition of TNFα-induced TAK1 phosphorylation was restored by OA treatment, as were E1A-mediated inhibition of IKK phosphorylation and the interaction between FOXO3a and βTrCP (Figure 6A). In a functional assay, E1A-induced chemosensitization was indeed decreased in MDA-MB-231 breast cancer cells by treatment with OA (Figure 6B).

To further define the mechanism involved in PP2A's inhibition of TAK1 activation, we examined whether PP2A directly binds to TAK1. As shown in Figure 6C, treatment with TNFα notably increased the interaction between TAK1 and IKK in 231/vector cells but not in 231/E1A cells. Moreover, PP2A formed a complex with TAK1 in 231/E1A cells and abolished the interaction between TAK1 and IKK (Figure 6C).

To confirm this novel binding between PP2A and TAK1, we treated 231/vector and 231/E1A cells with siRNA for catalytic subunit of PP2A, PP2A/C, (siPP2A/C) to target knockdown of PP2A/C protein and then measured the binding preference of TAK1. Treatment with siPP2A/C, but not with control siRNA, decreased E1A-induced PP2A/C expression (Figure 6D). The binding of TAK1 and PP2A in 231/E1A cells was decreased by treatment with siPP2A/C, and the formation of TAK1/IKK complex was increased (Figure 6D). E1A-mediated inhibition of phosphorylation of FOXO3a at Ser644 and the interaction between FOXO3a and βTrCP were also restored by treatment with siPP2A/C (Figure 6E). These findings indicated that

E1A-induced PP2A expression is required for regulation of TAK1/IKK signaling, FOXO3a phosphorylation, and chemosensitization.

DISCUSSION

E1A has many antitumoral activities and has been tested in multiple phase I and II clinical trials. Studies showed that although *E1A* gene therapy is safe and well tolerated, the tumor response to it is only modest(Hortobagyi et al., 1998; Hortobagyi et al., 2001; Madhusudan et al., 2004; Villaret et al., 2002; Yoo et al., 2001). However, E1A has been shown to induce sensitization to different categories of anticancer drug–induced apoptosis; therefore, one improvement that might render E1A more useful as an anticancer therapy is the combination of *E1A* gene therapy with conventional chemotherapy. It has been shown that paclitaxel, a front-line chemotherapeutic agent for the treatment of human breast and ovarian cancer, can up-regulate FOXO3a, subsequently increasing BIM (pro-apoptotic BH3-only protein) expression and ultimately decreasing cell survival and contributing to the tumor response to paclitaxel in paclitaxel-sensitive cells, such as MCF-7, in which FOXO3a expression level is already high. However, this phenomena has not been observed in MDA-MB-231 cells, which express low levels of FOXO3a (Sunters et al., 2003). In the current study, we found that E1A can stabilize FOXO3a in MDA-MB-231 cells, therefore sensitizing them to paclitaxel-induced apoptosis both in vitro and in vivo. This result provides evidence that the combination of *E1A* gene therapy and paclitaxel chemotherapy might be an effective way to treat those patients with paclitaxel-resistant tumors whose basal FOXO3a expression is low.

We found that E1A can protect FOXO3a from degradation by inhibiting its ubiquitination. Although previous studies showed that FOXO3a can be targeted by the proteasome pathway

after being phosphorylated by Akt or IKK(Brunet et al., 1999; Hu et al., 2004), its E3 Ub ligase was still unknown. In the current study, we found that βTrCP can physically bind to FOXO3a and mediate its degradation and that E1A stabilizes FOXO3a by inhibiting the binding of FOXO3a to βTrCP. βTrCP are the substrate recognition subunits of the Skp1 Cullin1 F-box protein E3 Ub protein ligases that can recognize specifically phosphorylated substrates and confer their ubiquitination. βTrCP plays a key role in the NF-κB signaling pathway by recognizing IKK-phosphorylated IκB and mediating its degradation (Fuchs et al., 2004). Our findings revealed a new substrate of βTrCP that requires phosphorylation by IKK. Previous studies showed that IKK can phosphorylate FOXO3a at serine 644 and cause FOXO3a nuclear exclusion(Hu et al., 2004). We found that this Ser644 phosphorylation mediated by IKK is also required for FOXO3a binding to βTrCP and for the further degradation induced by βTrCP. E1A prevents the binding of βTrCP to FOXO3A by inhibiting the IKK-mediated FOXO3a phosphorylation at Ser644. Akt phosphorylation also promotes FOXO3A degradation via a proteasome pathway (Plas and Thompson, 2003). In further investigating the influence of the Akt pathway on FOXO3a degradation, we found that Akt phosphorylation can also mediate the binding of FOXO3a to βTrCP, although that binding is much weaker than that mediated by IKK phosphorylation. This is, to our knowledge, is the first demonstration that βTrCP can recognize the substrate phosphorylated by Akt and functioning in the activated PI3K/Akt pathway. Since the binding of βTrCP to Akt-phosphorylated FOXO3a is so weak, it is possible that other E3 Ub ligases exist that can mediate the degradation of Akt-phosphorylated FOXO3a.

PI3K/Akt and IKK/NFκB are two pathways that play important roles in promoting cell survival, proliferation, and tumor progression. Both of them can phosphorylate FOXO3a and mediate its binding to βTrCP for degradation. In investigating their specific effect on the binding

of FOXO3a to βTrCP, we found that the binding mediated by IKK is independent of Akt, while the binding mediated by Akt is dependent on IKK. Therefore, we concluded that the activated IKK pathway plays a major role in the degradation of FOXO3a mediated by βTrCP. Previous studies showed that IKK can upregulate Akt phosphorylation at Ser473 (Sen et al., 2007). Ser473 phosphorylation on Akt facilitates its phosphorylation on Thr308 and is important for the recognition and activation of Akt by phosphoinositide-dependent kinase 1 (PDK1). Thus, Akt-mediated binding of FOXO3a to βTrCP may be dependent on the activation of Akt through up-regulation of Ser473 by IKK.

IKK activation requires its phosphorylation by upstream kinases, including TAK1 (Sato et al., 2005; Takaesu et al., 2003; Wang et al., 2001), and phosphorylation plays a significant role in TAK1 activation (Singhirunnusorn et al., 2005). We found in this study that E1A inhibits FOXO3a phosphorylation and binding to βTrCP by preventing TAK1 activation and its effect on IKK activation. It was previously shown that TRAF6 and RIP1 can activate TAK1 and lead to IKK phosphorylation and activation (Kelliher et al., 1998; Ninomiya-Tsuji et al., 1999; Ting et al., 1996). However, overexpression TRAF6 or RIP1 in E1A-stable cell lines could not restore TAK1 activation and mediate FOXO3a degradation (data not shown), indicating that prevention of TAK1 activation by E1A is not mediated by those two upstream activators. Thus, it is possible that the inactivation is mediated by the phosphatase. PP2A phosphatase activity is enhanced in E1A-expressing cells through E1A-mediated up-regulation of PP2A/C expression, which results in repression of Akt activation (Liao and Hung, 2004). We found that TAK1 is a novel target of PP2A/C. Therefore, E1A-mediated up-regulation of PP2A/C is involved in TAK1 inactivation and inhibits the binding of TAK1 to IKK, which abolishes IKK's function in phosphorylating FOXO3a, resulting in the stabilization of FOXO3a.

The activities of protein kinases are finely regulated by phosphorylation and dephosphorylation, however, little is known about the dephosphorylation and respective protein phosphatase involved in the regulation of TAK1. Protein phosphatase 2A (PP2A) is a ubiquitously expressed protein serine/threonine phosphatase accounts for the tumor suppression activity in eukaryotic cells. Mutation of PP2A was found in human breast, colon, and lung cancers and melanoma (Schonthal, 2001). In addition, a variety of mechanisms for inactivating PP2A were found to be involved in transformed cells. PP2A can be inhibited by the small T antigen of the DNA tumor virus SV40 (Arroyo and Hahn, 2005), or by upregulation of the c-Myc-specific inhibitor CIP2A (Junttila et al., 2007), or through the upregulation of SET protein by BCR/ABL oncogene (Neviani et al., 2005). It was previously showed that PP2A phosphatase activity is enhanced in E1A-expressing cells through E1A-mediated up-regulation of catalytic subunit PP2A/C expression, which results in repression of Akt activation (Liao and Hung, 2004). Besides Akt, PP2A has many other substrates including c-Myc (Junttila et al., 2007) and RalA (Sablina et al., 2007), therefore, suppresses PI3K/Akt and ERK group of MAP kinase pathways and RalA activity. It seems that important substrates of PP2A are downstream components of Ras signaling and an important action of PP2A in tumor suppression is to antagonize signaling downstream of activated Ras (Mumby, 2007). We found in this paper that TAK1 is a novel target of PP2A/C, which is not a component in Ras signaling, but rather, in another important signaling pathway—IKK pathway. E1A-mediated up-regulation of PP2A/C is involved in TAK1 inactivation and inhibits the binding of TAK1 to IKK, which abolishes IKK's function in phosphorylating FOXO3a, resulting in the stabilization of FOXO3a. Therefore, E1A-mediated PP2A activation suppresses both Akt and IKK pathways, among which the suppression of IKK pathway is involved in the stabilization of FOXO3a.

In summary, we found that FOXO3a is critical for E1A-mediated chemosensitization. On the basis of our findings, we propose a model in which E1A stabilizes FOXO3a by inducing the expression of PP2A/C, which inhibits the activation of IKKβ through binding and inactivation of TAK1, therefore inhibiting IKKβ-mediated FOXO3a phosphorylation at Ser6444 and preventing βTrCP-induced FOXO3a degradation (Figure 7).

EXPERIMENTAL PROCEDURES

Cell Lines, DNA Constructs, and Antibodies

Cell lines MDA-MB-231, MDA-MB-453, MDA-MB-468, SKOV3, HeLa and NIH3T3 cell were purchased from ATCC and grown in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal bovine serum. *Ikkβ*$^{-/-}$ MEF cells have been described previously (Lee et al., 2007). The human breast cancer cell line MDA-MB-231 and its E1A/vector-stable transfectants and the human ovarian cancer cell line SKOV3-ip1 and its E1A/vector-stable transfectants have been described previously (Meric et al., 2000; Yu et al., 1993). The transfectants were grown under the same conditions as the controls, except that G418 was added to the culture medium. siFOXO3a stable transfectants were selected by the use of blasticidin S.

Plasmids E1A (Yu et al., 1990), FOXO3a-HA, IKKβ (Hu et al., 2004), βTrCP-myc, βTrCP△F-myc (kindly provide by Dr. Serge Y. Fuchs, University of Pennsylvania), PP2A/A, PP2A/C (Liao and Hung, 2004), and TAK1-HA (Blonska et al., 2005) were described previously. FOXO3a siRNA plasmids were kindly provided by Dr. A Toker (Storz et al., 2005), and βTrCP siRNA plasmids, by Dr. Serge Y. Fuchs (Tang et al., 2005).

We used antibodies to Flag (F3165; Sigma), Myc (11667203001; Roche), HA (11666606001; Roche). The monoclonal antibody used against the E1A proteins was M58

(Pharmingen). The following were obtained as indicated: FOXO3a (SC-11351; Santa Cruz Biotechnology), IκBα (SC-371; Santa Cruz Biotechnology), pIκBα (S32/S36) (9246; Cell Signaling Technology), IKKβ (2684; Cell Signaling Technology, or SC-7607; Santa Cruz Biotechnology), and pIKKβ (S181) (2681; Cell Signaling Technology). Rabbit antihuman PP2A/A and PP2A/C were purchased from CalBiochem. We also purchased the following from the suppliers indicated: ubiquitin (3936; Cell Signaling Technology), βTrCP (37-3400; Zymed, or SC-15354; Santa Cruz Biotechnology), TAK1 (SC-7967; Santa Cruz Biotechnology), pTAK1 (4531S; Cell Signaling Technology), and α-tubulin (T-5168; Sigma). Antibody to the Ser644 phosphorylation sites of FOXO3a was generated in collaboration with Bethyl Laboratories, Inc. Synthetic phosphorylated peptides representing the portion of FOXO3a around serine 644 were used as antigens for producing antibody. The antibody was then purified using a phosphopeptide column. BAY 117082 and parthenolide were purchased from Calbiochem. Recombinant human TNFα was purchased from Roche. MG132 was purchased from Sigma.

Immunoprecipitation and Western Blotting

Cells were washed twice with PBS, scraped into 500 μl of lysis buffer, and incubated on ice for 20 min. After centrifugation at 14,000 × *g* for 10 min, 1.5 mg of each supernatant was preincubated with 2 μg of immunoglobulin G and 50 μl of protein G for 1 h at 4°C. Immunoprecipitation was performed overnight with 2 μg antibody and 50 μl of protein G. The immunocomplex was washed five times with lysis buffer, dissolved in loading buffer, subjected to SDS-PAGE, and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20 and incubated with primary antibodies, followed by secondary antibodies (Jackson ImmunoResearch Laboratories). The immunoblots were visualized with enhanced chemiluminescence (Amersham).

Peptide Competition

The synthetic peptides used to target Ser644 phosphorylation were derived from the antigen peptides for antibody induction by linking the amino-terminal end to the TAT-1 nuclear-localization sequence (GRKKRRQRRR) for enhance the permeability of this peptide into cells (Ammosova et al., 2005), which were prepared and provided by Bethyl Laboratories. One hundred micrograms of FOXO3a peptide or scrambled peptide was incubated with cells for 6 h, and then the cells were treated with TNFα (20 ng/ml) for 30 min. Cell lysates were collected and subjected to immunoprecipitation assay.

Paclitaxel-Induced Cell Death

Cells were treated with 20 nM paclitaxel and incubated for 24 h. Aliquots of 1×10^6 cells were collected and washed once with ice-cold PBS and then fixed with ice-cold 70% ethanol overnight. After fixation, cells were washed with PBS to remove residual ethanol, pelleted, and resuspended in PBS containing 50 μg/ml of propidium iodide (Sigma). Staining was performed at 4°C for at least 30 min, and samples were analyzed using an Epics Profile flow cytometer (Coulter) in the Core Facility at The University of Texas M. D. Anderson Cancer Center.

Orthotopic Breast Tumor Growth Assay

Six-week-old female SCID mice were supplied by the animal center of Tzu Chi University, Hualien, Taiwan. Mice were orthotopically inoculated with tumor cells into the mammary fat pad as described previously (Lee et al., 2007). Tumor development was followed in individual animals (eight per group) by measuring tumor length (L) and width (W) with calipers every 3 days. Tumor volume was calculated with the formula $LW^2/2$. All animal work and care was

performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of China Medical University.

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FIGURE LEGENDS

Figure 1. FOXO3a Is Critical for E1A-Mediated Chemosensitization

(A) E1A-expressing vector (E1A) or control vector (vector) was transfected into different types of cells, followed by analysis of E1A and FOXO3a protein expression by Western blot analysis. α-Tubulin was used as the internal protein loading control. (B) E1A-induced FOXO3a expression was required for E1A-mediated chemosensitization. Upper panel: Chemosensitization of E1A–expressing cells or vector control cells (231/E1A and 231/vector; ip-1/E1A and ip-1/vector) stably transfected with siFOXO3a or control siRNA as analyzed by the DNA flow cytometry assay. Each type of transfected cell was treated with 20 nM paclitaxel (Taxol) for 24 h and assayed in three experiments. The columns are the mean values from the three independent experiments, and the error bars are the corresponding upper 95% confidence intervals. Asterisks denote a statistically significant difference compared with values of bar 1 (*$p<0.05$, two-tailed Student's *t* test. E1A-dependent chemosensitization was overturned by siFOXO3a to a significant degree, as indicated by the # symbol. Lower panel: Expression of FOXO3a was analyzed by Western blotting. Data are representative of three independent experiments. (C) Tumor volume of orthotopic xenograft tumors formed by MDA-MB-231/vector cells or MDA-MB-231/E1A cells stably transfected with either control siRNA (siControl) or FOXO3a siRNA (siFOXO3a). Each column represents the mean ± SD of eight primary tumors. *$p<0.05$ versus bar 2 values, by two-tailed Student's *t* test. E1A-dependent chemosensitization was overturned by siFOXO3a to a significant degree, as indicated by the # symbol. (D) Expression of FOXO3a protein and E1A was examined by immunoblotting assays using MDA-MB-231/vector tumors or MDA-MB-231/E1A tumors stably transfected with either control siRNA (siControl) or FOXO3a siRNA (siFOXO3a).

Figure 2. E1A Prevents Ubiquitin-Dependent Proteolysis of FOXO3a

(A) Determination of the protein stability of FOXO3a in MDA-MB-231/vector cells and MDA-MB-231/E1A cells. The 231/vector cells and 231/E1A cells were treated with 100 μg/ml cycloheximide (CHX) for the indicated times. Total protein was isolated, and expression of FOXO3a was analyzed by Western blotting assay and quantified (bottom). The results are representative of at least three independent experiments. Error bars, SD. (B) Upper panel: Lysates of 231/vector or 231/E1A cells transfected with HA-ubiquitin (HA-Ub) were treated with the proteasome inhibitor MG132 with or without TNFα (20 ng/ml) and were analyzed using immunoprecipitation/immunoblotting. Lower panel: Lysates of 231/vector or 231/E1A cells transfected with HA-ubiquitin were subjected to Western blotting.

Figure 3. βTrCP Is Involved in E1A-Induced FOXO3a Induction

(A) Expression of E1A disrupted the interaction between βTrCP and FOXO3a. Lysates of MDA-MB-231/vector or MDA-MB-231/E1A cells left untreated or treated with TNFα (20 ng/ml) were analyzed by immunoprecipitation/immunoblotting (IP/IB). (B and C) Lysates of HeLa cells cotransfected with the indicated vectors and left untreated or treated with TNFα were subjected to IP/IB (anti-HA/anti-Myc). (D) IKKβ was required for the interaction between βTrCP and FOXO3a. Lysates of HeLa cells cotransfected with the indicated vectors were treated as described in panels B and C. (E) Knockdown of βTrCP expression by βTrCP-specific siRNAs increased FOXO3a expression. The 231/vector cells and 231/E1A cells were treated with siβTrCP1, siβTrCP2, or control siRNA for 48 h. Total protein was isolated, and expression of FOXO3a and βTrCP was analyzed by Western blotting. (F) Knockdown of βTrCP expression by

βTrCP-specific siRNAs disrupted the interaction between βTrCP and FOXO3a. Lysates of 231/vector or 231/E1A cells left untreated or treated with siβTrCP in the presence of MG132 (5 μg/ml) were analyzed by IP/IB (anti-FOXO3a/anti-βTrCP).

Figure 4. E1A Inhibits IKKβ-Mediated FOXO3a Phosphorylation at Ser644 and Prevents βTrCP-Induced FOXO3a Degradation

(A) Akt phosphorylation sites of FOXO3a (Thr32, Ser253, and Ser315) and IKKβ phosphorylation sites of FOXO3a (Ser644) were mutated to glutamine to mimic the phosphorylated status of FOXO3a. Lysates of HeLa cells cotransfected with GFP-FOXO3a/3E mutant (all three Akt phosphorylation sites of FOXO3a were mutated to glutamine) or GFP-FOXO3a/S644E mutant with Myc-βTrCP were subjected to immunoprecipitation/immunoblotting (IP/IB) (anti-GFP/anti-Myc). (B) The same phosphorylation sites of FOXO3a described in panel A were mutated to alanine to mimic the inactivated status of FOXO3a. Lysates of HeLa cells cotransfected with indicated expressing vectors were subjected to IP/IB (anti-GFP/anti-Myc). (C) Lysates of MDA-MB-231 cells treated with MG132 and with or without TNFα (20 ng/ml) in the presence of synthetic peptides were subjected to IP/IB (anti-FOXO3a/anti-βTrCP). (D) Lysates of wild-type (WT-MEF) or IKKβ knockout mouse embryonic fibroblasts ($IKK\beta^{-/-}$-MEF) cotransfected with the indicated vectors and treated or untreated with TNFα (20 ng/ml) were analyzed by IP/IB (anti-HA/anti-Myc). (E) Lysates of 231/vector and 231/E1A cells treated with MG132 and with or without TNFα (20 ng/ml) were subjected to IP/IB (anti-FOXO3a/anti-FOXO3a-pS644 and anti-βTrCP) analysis and Western blotting (pIκB and βTrCP). (F) Lysates of 231/vector and 231/E1A cells transfected with or without HA-IKKβ were subjected to IP/IB (anti-FOXO3a/ anti-FOXO3a-pS644 and anti-

βTrCP) analysis. (G) HeLa cells were transfected with E1A or HA-IKKβ in combination with IKKβ inhibitors (parthenolide, 60 μM, 1 hour pretreatment, and BAY117082, 30 μM, 1 hour pretreatment) or paclitaxel (Taxol; 20 nM), and then chemosensitization was analyzed by the detection of cell death using DNA flow cytometry. The columns are the means of three independent experiments, and the error bars are the corresponding upper 95% confidence intervals.

Figure 5. Down-regulation of TAK1 Activity Is Critical for E1A-Mediated FOXO3a Stabilization and Chemosensitization

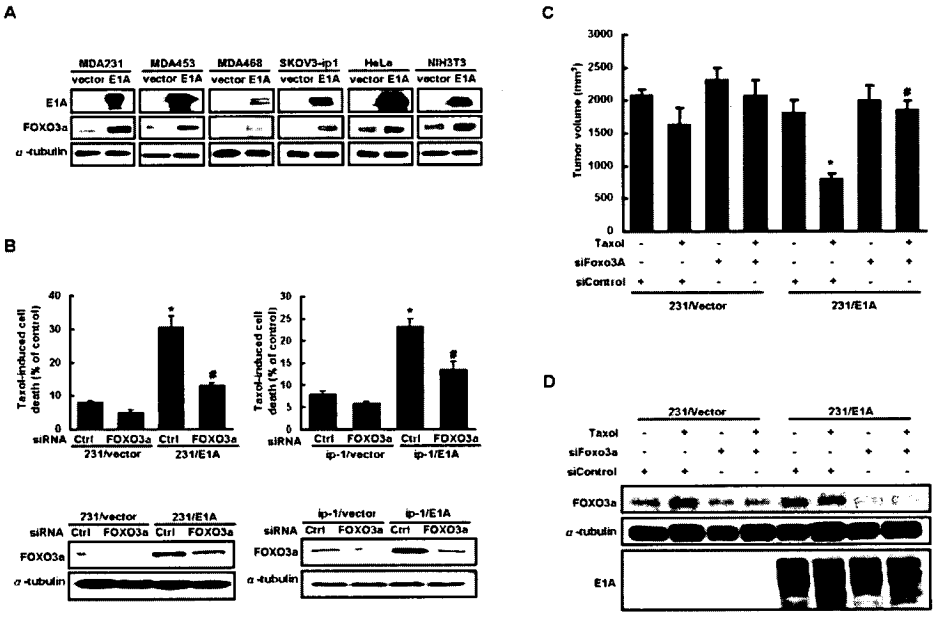
(A) Lysates of MDA-MB-231/vector and MDA-MB-231/E1A cells left untreated or treated with TNFα (20 ng/ml) were subjected to Western blotting to analyze the phosphorylation of IKK and TAK1. (B) Lysates of 231/vector and 231/E1A cells transfected with or without HA-TAK1 were subjected to Western blotting to analyze the expression of FOXO3a and phosphorylated IKK protein. (C) Lysates of 231/vector and 231/E1A cells transfected with or without HA-TAK1 were subjected to immunoprecipitation/immunoblotting (IP/IB) (anti-FOXO3a/anti-FOXO3a-pS644 and anti-βTrCP) analysis. (D) 231/E1A and 231/vector cells were transfected with HA-TAK1 or control vector and then analyzed by DNA flow cytometry. Each type of transfected cell was treated with 20 nM paclitaxel for 24 h. The columns are the means of three independent experiments, and the error bars are the corresponding upper 95% confidence intervals. *$p<0.05$ versus bar 1 values, by two-tailed Student's *t* test. E1A-dependent chemosensitization was overturned to a significant degree by transfection with HA-TAK1, as indicated by the # symbol.

Figure 6. E1A-Induced PP2A Expression Is Required for Regulation of IKK Signaling, FOXO3a Phosphorylation, and Chemosensitization

(A) Western blot analyses of the phosphorylation of TAK1 and IKK and the interaction between FOXO3a and βTrCP in MDA-MB-231/vector and MDA-MB-231/E1A cells left untreated or treated with TNFα (20 ng/ml) and 10 nM okadaic acid (OA). Equal amounts of cell lysates were resolved by SDS–PAGE, transferred to PVDF membranes, and probed with specific anti-phosphorylated TAK1 and IKK antibodies and anti-TAK1 and IKK antibodies. The results are representative of at least three independent experiments. The cell lysates were also subjected to immunoprecipitation/immunoblotting IP/IB (anti-FOXO3a/anti-βTrCP) analysis. (B) 231/E1A and 231/vector cells were left untreated or treated with OA (10 nM) combined with 20 nM paclitaxel (Taxol) for 24 h and then analyzed for chemosensitization by DNA flow cytometry. The columns are the means of three independent experiments, and the error bars are the corresponding upper 95% confidence intervals. *$p<0.05$ versus the values of bar 1, by two-tailed Student's *t* test. E1A-dependent chemosensitization was overturned to a significant degree by treatment with OA, as indicated by the # symbol. (C) Lysates of 231/vector and 231/E1A cells left untreated or treated with 20 ng/ml of TNFα were subjected to IP/IB (anti-TAK1/anti-IKK and anti-PP2A/C) analysis. (D) E1A-induced PP2A/C expression was required for E1A-mediated signaling. Lysates of 231/vector and 231/E1A cells transfected with siPP2A/C or control siRNA were subjected to IP/IB (anti-TAK1/anti-IKK and anti-PP2A/C) analysis and Western blotting (PP2A/C and PP2A/A). (E) Lysates of 231/vector and 231/E1A cells transfected with siPP2A/C or control siRNA were subjected to IP/IB (anti-FOXO3a/anti-FOXO3a-pS644 and anti-βTrCP) analysis.

Figure 7. A model of molecular mechanisms involved in E1A-mediated chemosensitization. A model in which E1A stabilizes FOXO3a by inducing the expression of PP2A/C, which inhibits the activation of IKKβ through binding and inactivation of TAK1, therefore inhibiting IKKβ-mediated FOXO3a phosphorylation at Ser644 and preventing βTrCP-induced FOXO3a degradation, and thus inducing chemosensitization.

Figure 1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Taxol-induced cell death (% of control)
siRNA Ctrl FOXO3a Ctrl FOXO3a
231/vector
231/E1A
ip-1/vector
ip-1/E1A
C
Tumor volume (mm³)
Taxol
siFoxo3A
siControl
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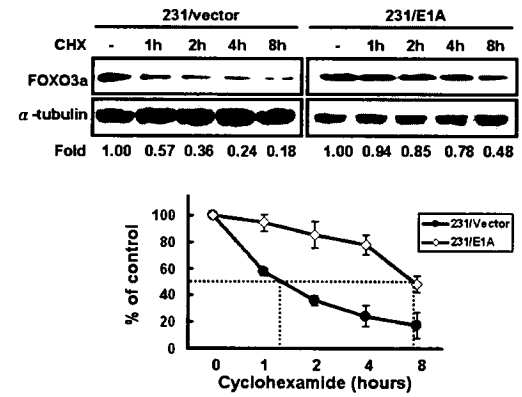
E1A

Figure 2

A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FOXO3a
α-tubulin
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60
40
20
0
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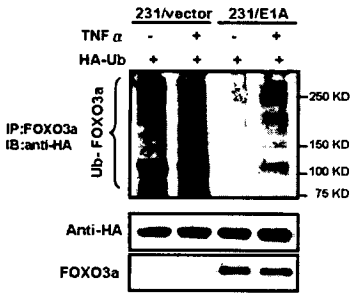
231/E1A
0 1 2 4 8
Cyclohexamide (hours)

B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HA-Ub + + + +
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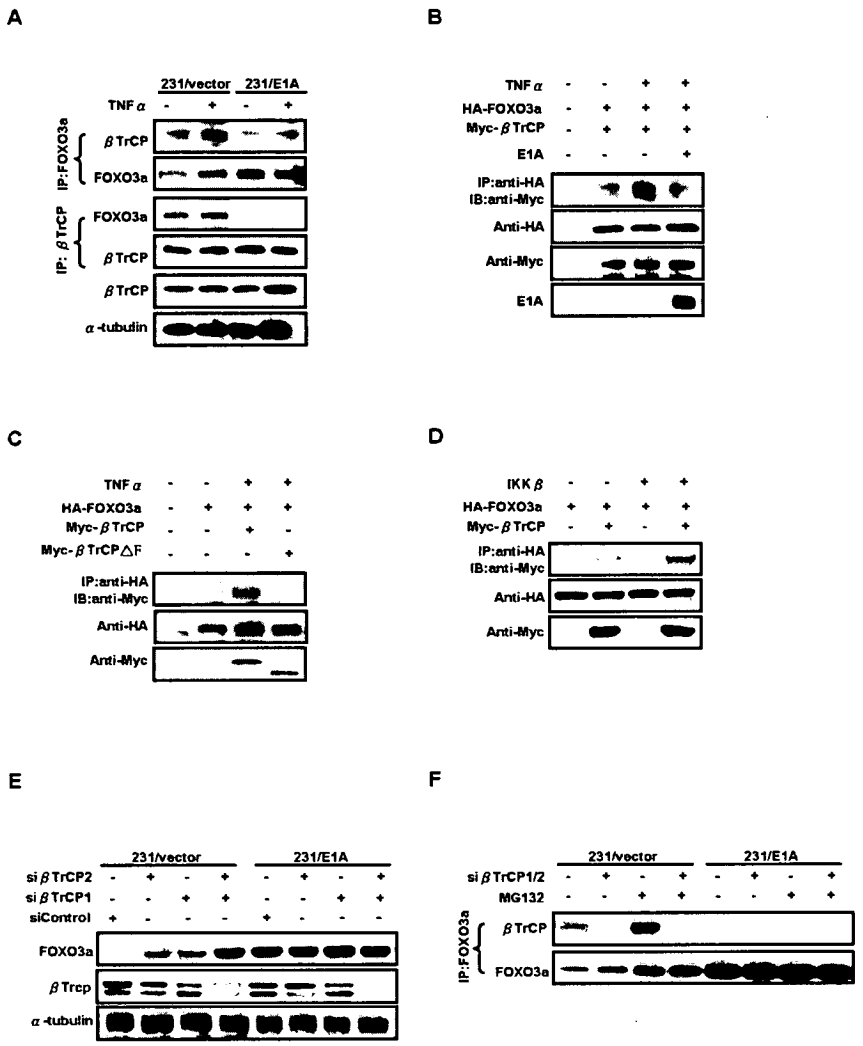
150 KD
100 KD
75 KD
Anti-HA
FOXO3a

Figure 3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231/E1A
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βTrCP
FOXO3a
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βTrCP
α-tubulin
B
TNFα
HA-FOXO3a
Myc-βTrCP
E1A
IP:anti-HA
IB:anti-Myc
Anti-HA
Anti-Myc
E1A
C
TNFα
HA-FOXO3a
Myc-βTrCP
Myc-βTrCPΔF
IP:anti-HA
IB:anti-Myc
Anti-HA
Anti-Myc
D
IKKβ
HA-FOXO3a
Myc-βTrCP
IP:anti-HA
IB:anti-Myc
Anti-HA
Anti-Myc
E
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231/E1A
siβTrCP2
siβTrCP1
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βTrcp
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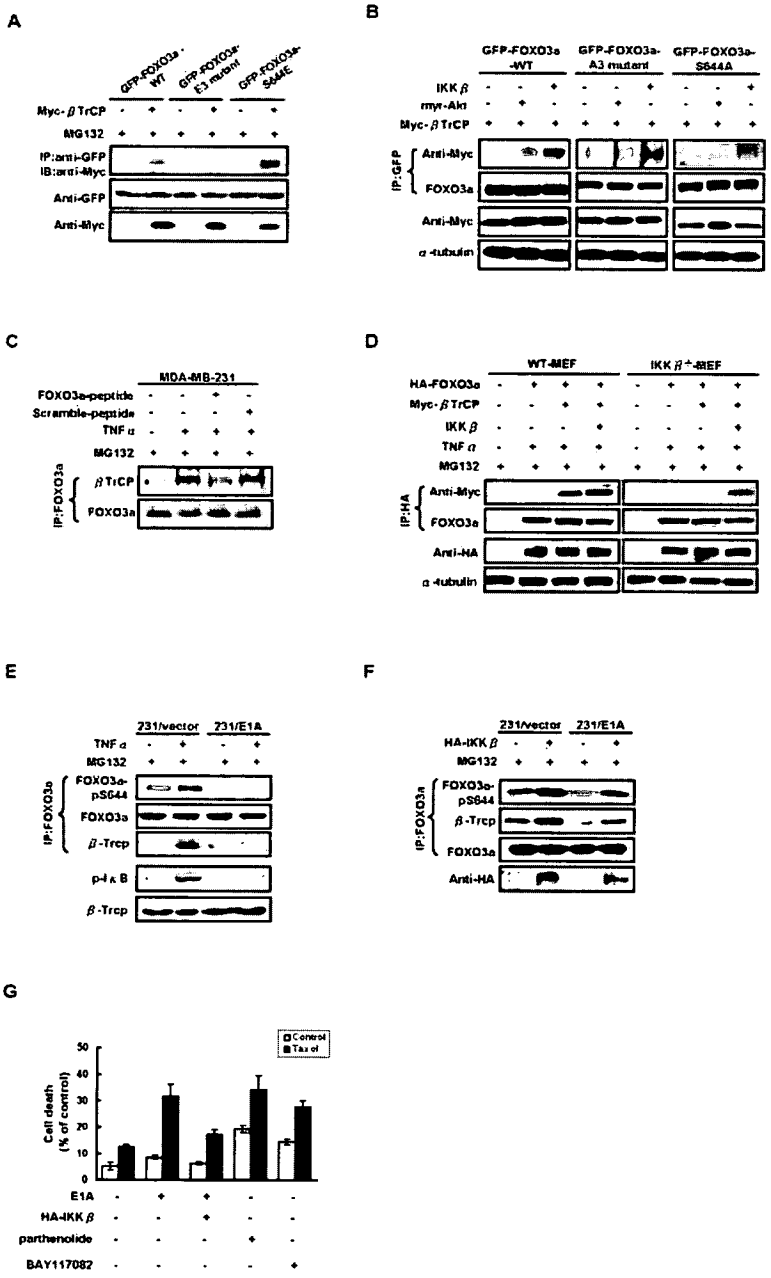
Figure 4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Anti-GFP
Anti-Myc
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Anti-Myc
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Scramble-peptide
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βTrCP
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IKKβ-/-MEF
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Myc-βTrCP
IKKβ
TNFα
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FOXO3a
Anti-HA
α-tubulin
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TNFα
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β-Trcp
FOXO3a
Anti-HA
G
Control
Taxol
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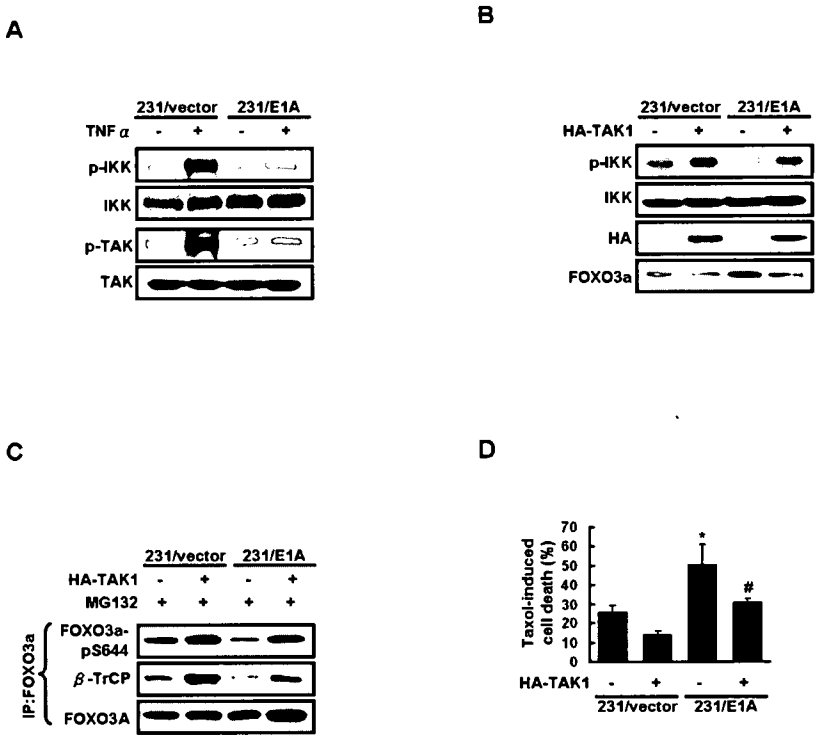
BAY117082

Figure 5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FOXO3a-pS644
β-TrCP
FOXO3A
D
Taxol-induced cell death (%)
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HA-TAK1
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Figure 6

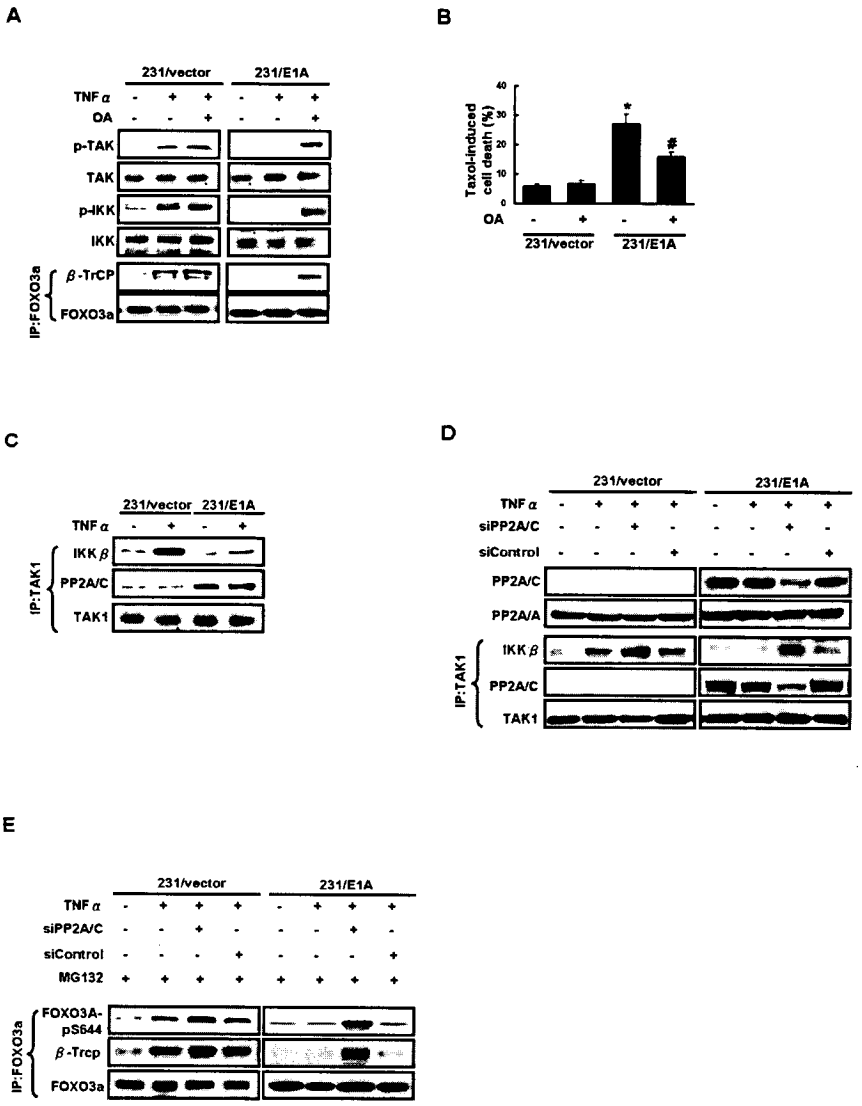
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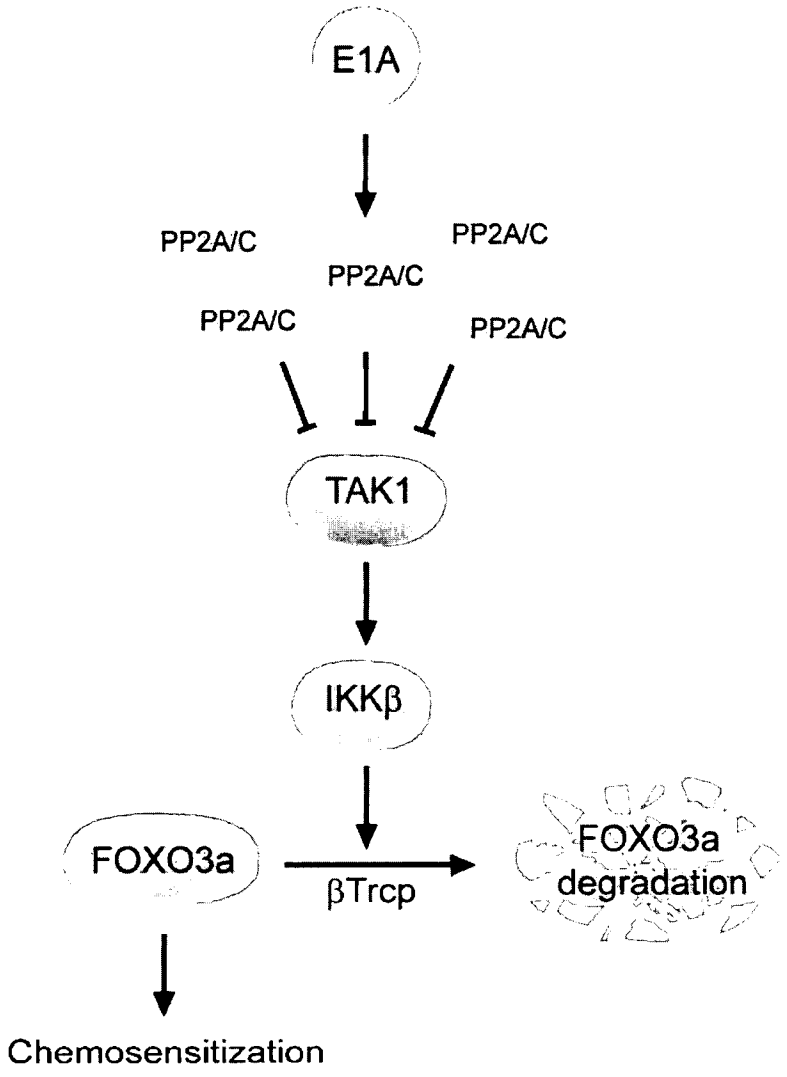
Figure 7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FOXO3a
degradation
Chemosensitization