國立台灣大學醫學院免疫學研究所

碩士論文

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Master Thesis

研究 mTOR 分子於胃幽門螺旋桿菌調控胃上皮細胞對 TRAIL 細胞凋亡訊息傳導所扮演之角色

Study the role of mTOR in regulation of *Helicobacter pylori*-induced TRAIL apoptosis signaling in Human gastric epithelial cells

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本論文係林怡孜君（學號 R05449002）在國立臺灣大學醫學院免疫學研究所完成之碩士學位論文，於民國 107 年 7 月 19 日承下列考試委員審查通過及口試及格，特此證明

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中文摘要

胃幽門螺旋桿菌(*Helicobacter pylori*)是一種世界上常見的細菌感染源之一，主要棲息在人體胃部，並且已被發現與許多胃部疾病的發生有關，例如胃炎、胃潰瘍、黏膜層淋巴瘤、胃癌。其中，過去研究發現感染胃幽門螺旋桿菌的表皮細胞其細胞凋亡的程度增加與胃幽門螺旋桿菌所導致的胃部病變有關。先前實驗室的研究顯示，TRAIL 能誘導感染胃幽門螺旋桿菌的胃上皮細胞進行大量細胞凋亡，進一步我們發現胃幽門螺旋桿菌透過負調控 Akt 的磷酸化程度使得 AIP4 蛋白活性增加，導致重要的調控蛋白 FILPs 進行泛素化降解，進而細胞無法阻礙 TRAIL receptor 下游死亡複合物的形成，導致胃上皮細胞容易被 TRAIL 誘導細胞凋亡。於此篇我們更利用 AIP4 基因剃除後發現 AIP4 分子的確參與在調控胃幽門螺旋桿菌誘導之 TRAIL 細胞凋亡之訊息傳導途徑中。此外，在此篇研究中我們也探討了胃幽門螺旋桿菌如何調控 Akt 的磷酸化，我們發現胃幽門螺旋桿菌會降低 Akt 的上游分子 mTOR 的表現量，並且我們發現隨著感染時間增長，mTOR 的表現量降低與 Akt 去磷酸化程度、FLIPs 的表現量降低呈現正相關，因此我們推測 mTOR 可能也參與在胃幽門螺旋桿菌所誘發的胃上皮細胞 TRAIL 敏感性提高。我們同時也利用 mTOR 抑制劑與 mTOR siRNA 轉染證明若於胃上皮細胞抑制 mTOR 活性則會導致細胞內 FLIPs 表現量下降且同時誘導細胞對 TRAIL 敏感度提升，此現象如同模擬胃幽門螺旋桿菌感染的情況。再者，我們過度表現 mTORC2 於人類胃上皮細胞後發現藉由於細胞內增強 mTOR-Akt-FLIP 訊號傳遞路徑可以使人類胃上皮細胞抵禦胃幽門螺旋桿菌所誘發之 TRAIL 引起的細胞凋亡。在此篇研究中我們定義出 mTOR 分子於胃幽門螺旋桿菌的致病機制中扮演重要角色，並且提供其成為將來治療感染胃幽門螺旋桿菌的分子標的之可能性。

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Abstract

Helicobacter pylori (H. pylori) is one of the most common pathogens that inhabits in human stomach, which has been known for being related to amounts of gastric disease such as gastritis, peptic ulcers, gastric MALT lymphomas and gastric cancer. It has been reported that the etiology of *H. pylori* is associated with the enhancement of cell apoptosis in gastric epithelial cells. Previous study in our laboratory has shown that *H. pylori* confers susceptibility to TRAIL-mediated apoptosis in human gastric epithelial cells through decreased Akt phosphorylation, which results in activating the AIP4 and leads to FLIPs degradation sequentially. In this study, we have further demonstrated that knockout of AIP4 in AGS cells mitigated *H. pylori*-induced TRAIL-mediated apoptosis, which has clarified that Akt-AIP4-FLIPs pathway participated in *H. pylori*-induced TRAIL apoptosis signaling. Besides, in order to investigate how *H. pylori* regulates Akt phosphorylation state, we have searched for the upstream molecular of Akt and found mTOR expression decreased while *H. pylori* co-cultured with gastric epithelial cells. Moreover, we found that the augmentation of mTOR-Akt pathway by overexpressing mTORC2 in gastric epithelial cells reduced *H.pylori*-induced TRAIL-apoptosis signaling. In this study, we address the role of mTOR in *H. pylori*-induced TRAIL apoptosis signaling and provide a new insight for the pathogenesis of *H. pylori*.

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Introduction

1. Helicobacter pylori (H. pylori)

Helicobacter pylori (H. pylori) is one of the most common pathogens that infects approximately 50% of the population in worldwide (Tsai and Hsu, 2017). It was first cultivated successfully in 1982 by Australian researchers: Barry J. Marshall and Robin Warren. They have deciphered the crucial role of *H. pylori* in the aetiology of gastric inflammation, duodenal ulcer, gastric ulcer, and strongly associated with adenocarcinoma and lymphoma (Cave, 1997; Marshall and Warren, 1984). Hence, *H. pylori* was classified as class I carcinogen (IARC, 1994). *H. pylori* is a Gram-negative, spiral-shaped bacterium which inhabits in human stomach. The transmission of *H. pylori* is commonly through person-to-person by saliva, or be spread by fecal contamination of food and water (Aziz et al., 2015; Goh et al., 2011; Khalifa et al., 2010). Unlike other bacteria, *H. pylori* could survive in the harsh acidic environment in the stomach due to the production of urease that neutralizes stomach acid. Besides, *H.pylori* relies on its tail-like flagella to move around; and the helical shape facilitates *H. pylori* to penetrate the mucoid covered on gastric epithelial cells in order to colonize in stomach (Keilberg and Ottemann, 2016). Although the high prevalence of *H. pylori*, most of patients remain asymptomatic in the lifetime. The clinical consequences of *H.pylori*-infected patient are controlled by complicated interactions between the

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polymorphisms in host, bacteria, and environmental parameters (Backert et al., 2016; Kusters et al., 2006). The current treatment of *H. pylori* is taking advantage of antibiotics such as amoxicillin and clarithromycin in order to eradicate *H. pylori*. However, several cases have reported that *H. pylori* eradication therapy failed due to the development of resistance in bacteria (Garza-Gonzalez et al., 2014; Megraud, 2004, 2007). Therefore, clarifying the molecular mechanism inside the pathogenesis of *H. pylori* is imminent to meet the therapeutic needs.

2. Pathogenesis of *H. pylori*

The pathogenicity of *H. pylori* is mediated through the action of a variety of bacterial virulence factors on host epithelial cells including but not limited to, urease, vacuolating cytotoxin A (VacA), cag pathogenicity island, cytotoxin-associated gene A (CagA), peptidoglycan outer membrane proteins (e.g., BabA, SabA, OipA) and γ-glutamyltranspeptidase (GGT) (Peek and Crabtree, 2006; Polk and Peek, 2010; Teymournejad et al., 2017; Valenzuela et al., 2013). *H. pylori* infection dysregulates the intracellular signaling pathways. For instance, once the CagA protein translocated into cells, the NF-κB pathway and MAP kinase would be activated via EGFR activation (Diaz et al., 2018). Besides, VacA has been reported to disrupt the mitochondrial functions, stimulate cell apoptosis and exert cell autophagy (Ashktorab et al., 2008;

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Palframan et al., 2012; Raju et al., 2012; Terebiznik et al., 2009).

Moreover, it has been already observed that *H. pylori* enhances both proliferation and apoptosis rate in the gastric biopsy specimen from *H. pylori*-induced gastritis patients, which compared to the secondary gastritis patients or non-inflamed controls (Jones et al., 1999; Jones et al., 1997; Moss et al., 1996). The following research has also authenticated the main cause of *H. pylori*-induced gastric disease is through the alteration of the balance between proliferation and apoptosis leading by *H. pylori* infection (Peek and Blaser, 2002). Furthermore, the apoptosis augmented by *H. pylori* stimulates the proliferation of gastric epithelial cells to maintain the integrity of epidermal barrier. This may increase the gene mutation rate in hyper-proliferative epithelial cells, and thus facilitate cells to become cancer-prone (Ashktorab et al., 2008; Xia and Talley, 2001). As mention above, the outcome of *H. pylori*-infection hinges on the bacteria virulence factors, host gastric mucosal factors and environment (Backert et al., 2016; Kusters et al., 2006). Previous reports have shown that gastric-infiltrating T cells are selectively increase during infection, especially resemble the phenotype of T helper 1 (Th1). Those T cells secret Th1 cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) in order to promote other pro-inflammatory cytokines release, which further enhances *H. pylori*-induced apoptosis (Bamford et al., 1998; Karttunen et al., 1995). On the other hand, *H. pylori* increases the expression of Fas in

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gastric epithelial cells. This also gives rise to the activation of apoptosis in gastric epithelial cells through additional Fas/FasL interaction with infiltrating T cells (Rudi et al., 1998). These finding indicate that *H. pylori* is capable of modulating the immune response, therefore inducing apoptosis signaling cascade in gastric epithelial cells (Wang et al., 2000; Wu et al., 2004).

3. TRAIL-mediated apoptosis

TRAIL (tumor necrosis factor-related apoptosis inducing ligand; Apo2L) is discovered in 1995, which has been identified as a member of TNF superfamily through its sequence homology to TNF-α (Wiley et al., 1995). The canonical role of TRAIL is well-known for inducing cell apoptosis through interaction with TRAIL receptor 1 (TRAIL-R1; DR4) and TRAIL receptor 2 (TRAIL-R2; DR5). Upon TRAIL binding to TRAIL-R1 or TRAIL-R2, the intracellular death domain (DD) of TRAIL-R adopts a conformation change, which endows the ability to recruit the intracellular adaptor molecule FAS-associated death domain protein (FADD). FADD further recruits the initiator caspases-8/10 via their death effector domain (DED) and forms the death-inducing signaling complex (DISC), which results in active the caspase-8/10 afterward(von Karstedt et al., 2017). Although TRAIL is enable to induce apoptosis in a variety of transformed cell lines in vitro, the normal primary cells exhibit resistance

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against TRAIL (Ozoren and El-Deiry, 2002).

There are several mechanisms for cells to withstand the TRAIL-inducing apoptosis (Lu et al., 2006). For instance, the FLICE-like inhibitory protein (FLIP, also known as CFLAR) is one of the negative regulator of TRAIL-mediated apoptosis. FLIP is a death effector domain-containing protein that could be recruited to DISC. Since it is a caspase-8 homologue that could compete with caspase-8 for binding to FADD, it impedes the activation process of caspase-8 and prevents TRAIL-mediated apoptosis (Hersey and Zhang, 2001). FLIP has 13 distinct spliced variants, three of which are expressed as proteins: 55 kda long form FLIP ($FLIP_L$), 25kda short form FLIP ($FLIP_s$), 24 kda form $FLIP_R$ (Bagnoli et al., 2010; Micheau, 2003; Safa et al., 2008; Safa and Pollok, 2011). The difference between FLIP isoforms indicates distinct regulatory role of $FLIP_L$ and $FLIP_s$. $FLIP_s$ inhibits TRAIL-induced DISC formation and caspase-8 activation, hence inhibit apoptosis (Poukkula et al., 2005; Yu and Shi, 2008). While $FLIP_L$ is controversial for preventing apoptosis due to the dual functions: when expressed at high-levels, it inhibits Fas-induced caspase-8 activation; however, it enhances caspase-8 activation when the expression level is low (Budd et al., 2006; Li et al., 2006).

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4. *H. pylori* enhances TRAIL-induced apoptosis signaling in gastric epithelial cells

Previously, our laboratory has shown that gastric infiltrating T cells express TRAIL by the analysis of gastric biopsy of *H. pylori*-infected patients. Further study have demonstrated that *H. pylori* enhances TRAIL-induced apoptosis (Wu et al., 2004); the molecular mechanism has not being fully decoded, nevertheless. We already ascertained the *H. pylori*-induced TRAIL-mediated apoptosis in gastric epithelial cells is dependent on activation of downstream signaling of caspase-8, and further transmit the death signals to mitochondria to break the resistance to apoptosis. Furthermore, we found *H. pylori* raises the TRAIL-sensitivity in gastric epithelial cells through downregulating FLIPs, which sparked off the initiation of caspase signaling cascade via DISC, and eventually undergo apoptosis (Lin et al., 2014; Tsai and Hsu, 2017). Recently the emerging clues in our laboratory point out that *H. pylori* might decrease FLIPs expression through dephosphorylating Akt. However, how *H. pylori* interferes intracellular Akt phosphorylation state still remains a puzzle.

5. The Akt pathway in regulation of FLIP$_s$ expression

The Ser and Thr kinase Akt, which is also known as (protein kinase B), was first identified as an oncogene and served as the downstream of phosphoinositide 3 kinase

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(PI3K) (Bellacosa et al., 1991; Staal, 1987). Akt is involved in several cellular processes including cell survival, proliferation and growth (Brunet et al., 1999; Fruman et al., 1999; Kodaki et al., 1994; Manning and Toker, 2017). Activation of Akt is a multistep process that involves both membrane translocation and phosphorylation. Mechanistically, class I PI3K transduces upstream signaling from receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) by phosphorylating the phosphoatidylinositol-4,5-bisphophate (PI-4,5-P_2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP_3) (Auger et al., 1989; Whitman et al., 1988). PIP_3 serves as a critical lipid second messenger that recruits Akt and Pyruvate Dehydrogenase Kinase 1(PDK1) to dock in the plasma membrane (Toker, 2012; Toker and Cantley, 1997; Vanhaesebroeck and Waterfield, 1999). PDK1 phosphorylates the activation loop of Akt at Thr308 and the full activation is accomplished by mTOR Complex 2 (mTORC2) on phosphorylating the Ser473 site of Akt (Sarbassov et al., 2005; Saxton and Sabatini, 2017). In addition, there are several phosphatases which are known for controlling Akt activation such as PIP_3 phosphatase PTEN(Phosphatase and tensin homolog) (Maehama and Dixon, 1998), Akt Ser473 phosphatase PHLPP(Leucine-rich repeat protein phosphatase) and Thr308 phosphatase PP2A(Protein phosphatase 2)(Liao and Hung, 2004).

Akt has been reported to have an impact on the regulation of FLIPs expression. For

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instance, Akt is found to regulate FLIPs expression through ubiquitination by Itch (E3 Ubiquitin-Protein Ligase Itchy Homolog, also known as AIP4) in GBM cell lines and xenograft (Panner et al., 2009); another report also shows that Akt controls the FLIPs expression through administering the FLIPs mRNA accumulation in mono/polyribosome (Panner et al., 2005) or directly affects the FLIP transcriptional level (Nam et al., 2003). Akt upregulation was also observed with enhanced FLIPs expression in tumor cells, thus exerting the resistance to TRAIL-mediated apoptosis to cells including the gastric cancer cell line (Nam et al., 2003).

6. mTOR signaling in regulation of FLIPs expression

mTOR (The mammalian target of rapamycin) was first identified as the direct target of rapamycin in 1994 (Brown et al., 1994; Sabatini et al., 1994; Sabers et al., 1995). mTOR is a serine/threonine protein kinase in the PI3K-related kinase (PIKK) family, which could form two distinct protein complex as mTOR complex 1 (mTORC1) and 2 (mTORC2) and participate in diverse signaling pathways. mTORC1 is composed of three core components: mTOR, Raptor (regulatory protein associated with mTOR), and mLST8 (mammalian lethal with Sec13 protein 8; GβL). The downstream signaling network of mTORC1 includes protein synthesis, cellular metabolism and protein turnover. While mTORC1 regulates cell growth and metabolism, mTORC2 masters the

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proliferation and cell survival through phosphorylating several protein kinase of the AGC (PKA/PKG/PKC) family, especially known for activating Akt, a key effector of insulin/PI3K signaling (Saxton and Sabatini, 2017). There are several evidences demonstrated that mTOR regulates c-FLIP expression. In the study of bladder cancer cells, utilizing metformin, which inhibits mTOR with activating autophagy, downregulates c-FLIP levels and contributes toward the enhancement of TRAIL-sensitivity (Zhang et al., 2014). Another report shows that mTOR controls FLIPs translation and TRAIL sensitivity in glioblastoma multiforme cells (Panner et al., 2005). In addition, mTORC2 was certified to be involved in c-FLIP degradation through E3 ubiquitin-protein ligase CBL(c-cbl) (Zhao et al., 2013). Taken together, these data support the importance of mTOR in mediating TRAIL-resistance in cells.

In the preliminary data, we have found the expression of mTOR decreased while co-cultured with *H. pylori*. Since there are several evidences show that mTOR is capable to regulate the FLIPs expression. This finding inspired us to explore the role of mTOR in *H. pylori*-mediated TRAIL sensitivity in human gastric epithelial cell line, especially focus on mTOR-Akt signaling axis.

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Rationales

It has been known that the pathogenesis of *H. pylori* is associated with induction of cell apoptosis. We have already demonstrated that *H. pylori* confers the susceptibility to TRAIL through decreasing of FLIPs expression, thus disabling gastric epithelial cells to prevent excess caspase-8 activation. Moreover, the preliminary results in our laboratory have revealed that *H. pylori* may negatively regulate Akt phosphorylation, which resulted in FLIPs ubiquitination and decreased the expression of FLIPs. Eventually, *H. pylori* enhanced TRAIL-mediated apoptosis in gastric epithelial cells. However, the regulation of Akt phosphorylation while *H. pylori* infection still remains unclear. We screened the upstream molecules that have been reported to affect the activation of Akt. Among these upstream molecules, we found that mTOR expression decreased during *H. pylori* infection. Base on this finding and the reports which have indicated that mTOR enabled to regulate c-FLIP expression, we consider mTOR as a possible molecule that is regulated by *H. pylori* and play a role in *H. pylori*-induced TRAIL apoptosis signaling in gastric epithelial cells.

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Materials and Methods

➢Materials

1. Cells

Human gastric epithelial cell line (AGS) were maintained in RPMI 1640 medium, supplemented with 10% Fetal Bovine Serum (FBS) and L-glutamine at 37℃ in 5% CO_2 incubator.

2. Bacteria

H. pylori strains ATCC 43504 and HM-9 were kindly provided by Dr. Ming-Shiang Wu. *H. pylori* was passaged on 5% sheep blood agar plates without antibiotics at 37℃ in micro-aerophilic incubator (10% CO_2, 5% O_2 and 85% N_2) for 1-2 days culture. For each experiment, *H. pylori* was scrapped from agar plates and re-suspended in 15 ml Brucella broth with antibiotics and cultured for 12-18hrs in micro-aerophilic incubator. The *H. pylori* was pellet at 3000rpm for 10 minutes and discarded the supernatants. The pellet was then re-suspended with 1ml 1X DPBS and measured the O.D. 600 value by spectrophotometer (1 O.D.= 10^9 CFU/ml). The re-suspended pellet was co-cultured in complete RPMI-1640 medium without antibiotics with cells.

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3. Antibodies

Primary Antibodies		
#9272	Anti-Akt	Cell signaling Technology, Danvers, USA
#9271	Anti-phospho-Akt (Ser473)	Cell signaling Technology, Danvers, USA
#9275	Anti-phospho-Akt (Thr308)	Cell signaling Technology, Danvers, USA
#12117	Anti-ITCH (D8Q6D)	Cell signaling Technology, Danvers, USA
#9746	Anti-Caspase-8 (1C12)	Cell signaling Technology, Danvers, USA
#56343	Anti-FLIP (D5J1E)	Cell signaling Technology, Danvers, USA
#2983	Anti-mTOR (7C10)	Cell signaling Technology, Danvers, USA
#2974	Anti-mTOR (Ser 2481)	Cell signaling Technology, Danvers, USA
#2114	Anti-Rictor (53A2)	Cell signaling Technology, Danvers, USA
#2280	Anti-Raptor (24C12)	Cell signaling Technology, Danvers, USA
#9234	Anti-phosphor-S6K (Thr389) (108D2)	Cell signaling Technology, Danvers, USA
#2708	Anti-p70-S6K (49D7)	Cell signaling Technology, Danvers, USA
#12860	Anti-mSin1 (D7G1A)	Cell signaling Technology, Danvers, USA
NB100-2331	Anti-LC3	Novus Biologicals, Colorado, USA
MAB1501	Anti-β-actin, clone C4	Merck Millipore, Billerica, USA
640941	APC-Annexin A5	BioLegend, San Diego, USA

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Secondary Antibodies		
#7074	Anti-rabbit IgG, HRP	Cell signaling Technology, Danvers, USA
#7076	Anti-mouse IgG, HRP	Cell signaling Technology, Danvers, USA

4. Recombinant protein

Recombinant TNF-related apoptosis-inducing ligand (TRAIL)

PeproTech, Rocky Hill, USA

5. Vector

peGFP-N2

peGFP-N2 with inserted human Akt cDNA by Shih-Chia Huang

peYFP-C1-mTOR Addgene Cambridge, Massachusetts, USA

pRK5-HA-YFP-rictor Addgene Cambridge, Massachusetts, USA

myc-Rictor corrected Addgene Cambridge, Massachusetts, USA

pcDNA3-Flag mTOR wt Addgene Cambridge, Massachusetts, USA

6. siRNA

SignalSilence® Akt siRNA I #6211 Cell signaling Technology, Danvers, USA

SignalSilence® Control siRNA #6568 Cell signaling Technology, Danvers, USA

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MISSION® mTOR siRNA (siRNA ID: SASI_Hs01_00203145)

Sigma-Aldrich, St Louis, Missouri, USA

MISSION® Rictor siRNA (siRNA ID: SASI_Hs02_00366683)

Sigma-Aldrich, St Louis, Missouri, USA

MISSION® Raptor siRNA (siRNA ID: SASI_Hs01_00048380)

Sigma-Aldrich, St Louis, Missouri, USA

MISSION® siRNA Universal Negative control

Sigma-Aldrich, St Louis, Missouri, USA

7. Transfection reagent

GenJet™ Plus DNA In Vitro Transfection reagent

SignaGen Laboratories, Maryland, USA

Lipofectamine™ 3000 reagent Invitrogen, California, Carlsbad, USA

Lipofectamine™ RNAiMAX reagent

Invitrogen, California, Carlsbad, USA

jetPRIME® in vitro DNA & siRNA transfection reagent

Polyplus transfection, Illkirch, France

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8. Medium and buffers

RPMI-1640 medium

Corning®RPMI-1640 culture medium containing 10% fatal bovine serum and L-glutamine with or without Antibiotic-Antimycotic (Gibco, USA)

Anaerobic Blood agar

CDC anaerobic blood agar which is supplemented with 5% sheep blood agar is purchased from BD biosciences, USA.

BBL broth

2.8% Brucella broth supplemented with 10% fatal bovine serum, 6μg/ml Vancomycin and 2μg/ml Amphotericin B

Triton X-100 lysis buffer

1% Triton-X, 50mM Tris-HCl, 5mM EDTA in ddH_2O

5X SDS sample buffer

250mM Tris-HCl (pH6.8), 5% β-mercapitalethanol, 0.02% Bromophenol blue, 30% glycerol, 10% SDS

10X running buffer

192mM Glycine, 25mM Tris-HCl, 1% SDS, pH=8.3

10X transfer buffer

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195mM Glycine, 240mM Tris-HCl, 1.185% SDS, pH=8.4. Dilute to 1X transfer buffer with 20% Methanol before usage.

10X washing buffer

100mM Tris-HCl (pH=7.4), 9% NaCl, 2% Tween-20

1X sorting buffer

1X HBSS(Ca^{2+}/Mg^{2+} free) with 1mM EDTA, 25mM HEPES, 2% FBS, pH= 7.0

1X AnnexinV binding buffer

Diluted from 10X AnnexinV binding buffer from BD biosciences, USA.

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9. Chemicals

Ammonium persulfate	Fluka, Switzerland
Amphotericin B	Sigma-Aldrich, St Louis, Missouri, USA
Acrylamide	Bio-Rad, Hercules, California, USA
β-mercapitalethanol	Sigma-Aldrich, St Louis, Missouri, USA
Bovine serum albumin	H.M. biologicals, 桃園市, 台灣
Brucella broth	Becton Dickinson, Franklin Lakes, New Jersey, USA
DMEM with high glucose	Gibco, USA
Dimethyl sulfoxide	Sigma-Aldrich, St Louis, Missouri, USA
Dulbecco's phosphate-buffered saline	Corning, Newark, USA
EDTA	Watson Biotech, 新北市, 台灣
Fetal bovine serum	Corning, Newark, USA
Glycerol	Sigma-Aldrich, St Louis, Missouri, USA
Glycine	Affymetrix, Santa Clara, California, USA
HEPES	Corning, Newark, USA
Kanamycin	MDBio, Inc., 台北市, 台灣
Metformin	Sigma-Aldrich, St Louis, Missouri, USA
MG132	Cell signaling Technology, Danvers, USA
Methanol	Merck, Darmstadt, Germany

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Miller’s LB broth power	Becton Dickinson, Franklin Lakes, New Jersey, USA
Opti-MEM® medium	Gibco, USA
Phosphatase inhibitor cocktail2	Sigma-Aldrich, St Louis, Missouri, USA
Propidium iodide	Sigma-Aldrich, St Louis, Missouri, USA
Phosphatase Inhibitor cocktail3	Sigma-Aldrich, St Louis, Missouri, USA
PP242	Selleckchem.com, Houston, USA
Prestained protein marker	BIOTOOLS，新北市，台灣
Protease inhibitor cocktail	Sigma-Aldrich, St Louis, Missouri, USA
Rapamycin	Selleckchem.com, Houston, USA
RIPA buffer	Omics Bio，新北市，台灣
Sheep blood	銳誠企業股份有限公司，新北市，台灣
Sodium bicarbonate	Affymetrix, Santa Clara, California, USA
Sodium chloride	Affymetrix, Santa Clara, California, USA
Sodium dodecyl sulfate	SERVA, Heidelberg, Germany
TEMED	Affymetrix, Santa Clara, California, USA
Torin1	Cayman Chemical, Michigan, USA
Tris-base	Affymetrix, Santa Clara, California, USA
Triton-X	Affymetrix, Santa Clara, California, USA
Trypan blue	H.M. biologicals，桃園市，台灣

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Tween-20	Affymetrix, Santa Clara, California, USA
Vancomycin	Sigma-Aldrich, St Louis, Missouri, USA
4-15% pre-cast gel	Bio-Rad, Hercules, California, USA

➢Methods

1. Cell death detection

(I) AnnexinV/ Propidium iodide apoptosis assay

Cells were cultured in 24-well (3×10^4 cells/well) overnight. For measuring *H.pylori* induced TRAIL-mediated apoptosis, cells were then treated with *H. pylori* (MOI=100) or not for 12 hours, following the recombinant TRAIL treatment (40ng/ml) for 6 hours. Cells were harvested by collecting the cell culture medium and the cells retained on the dish are washed by 1X DPBS, following accutase treatment for 15-20 minutes to obtain the residue cells on the dish. Centrifuge at 300×g, 5 minutes and discard the supernatant. The cell pellet was washed by 1X DPBS three times and re-suspended by AnnexinV binding buffer which contained AnnexinV-APC antibody and PI for staining 15 minutes in the dark. Before analyze by flow cytometry, dilute cells into appropriate volume by AnnexinV binding buffer.

(II) Cell death detection ELISA

Cells were cultured in 96-plate (1×10^4 cells/well) overnight. Then treated cells with *H.*

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pylori (MOI=100) or not for 12 hours, following the recombinant TRAIL treatment (40ng/ml) for 3 hours. Cells were harvested by centrifugation at 200×g for 10 minutes. Discard the supernatant carefully and lyse cells in 200μl 1x working solution of lysis buffer. After incubate with the lysis buffer for 30 minutes, centrifuge the cell lysate at 200×g for 10 minutes. The 20μl supernatant was transferred to streptavidin-coated microplate and incubate with 80μl prepared immunoreagents (1/20 anti-DNA-POD antibody, 1/20 anti-histone-biotin antibody and 18/20 incubation buffer) for 2 hours with shaking. After washing three times with the incubation buffer, add 100μl ABTS substrate solution into each well and keep the plate in the dark until development of the color was sufficient for photometric analysis. The reaction was determined by ELISA reader at 405nm.

2. Cell lysis

After cell were harvested and centrifuge into pellet, the appropriate volume of lysis buffer was added to re-suspend the cell pellet and incubate on ice for 20 minutes with vortexing occasionally. The cell lysate were centrifuge for 15 minutes at 15,000×g, 4℃. The proteins are in the supernatant and store the supernatant at -70℃.

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3. Western blot

Proteins were extracted and quantified by protein assay dye. Equal amount of proteins of each sample (20-35μg/ml) in SDS sample buffer were boiled for 5 minutes at 100℃. Load each sample into the gel and run the SDS-PAGE (6%-15%) at 100V, protein on the gel is transferred to PVDF membrane which was pre-treated in 100% methanol at 400mA by wet transfer system for 60-90 minutes. After transferring, put the PVDF membrane into the blocking buffer (5% non-fat milk or bovine serum albumin (BSA) in TBST buffer) and block for 1 hour. The membrane was incubated with indicated primary antibody diluted in blocking buffer at 4℃ overnight. After incubation, the membrane was washed with TBST buffer three times for 10 minutes and incubated with the certain horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 hour. Wash the membrane with TBST buffer three times for 10 minutes and develop the membrane by ECL system.

4. Real-time polymerase chain reaction (RT-PCR)

Total RNA form AGS cells was isolated using TRIzol (Invitrogen). cDNA was prepared following the instructions provided by manufacture (iScript^{TM} cDNA synthesis kit). The cDNA products were used to amplify target gene (mTOR, c-FLIP$_s$ and GAPDH) using SensiMix SYBR Bo-ROX kit (Bioline). Data were acquired with PikoReal

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(Thermo Fisher).

The PCR primers were:

Gene	Forward	Reverse
mTOR	5’-GCTTGATTTGGTTCCCAGGACAGT-3’	5’-GTGCTGAGTTTGCTGTACCCATGT-3’
FLIPs	5’- GCAGGGACAAGTTACAGGAATGT-3’	5’-GGACAATGGGCATAGGGTGT -3’
GAPDH	5’-GTGAACCATGAGAAGTATGACAA -3’	5’-CATGAGTCCTTCCACGATAC -3’

5. siRNA transfection

Different siRNA transfection reagent was used to conduct experiments under the instructions provided by manufacturer. For Akt knockdown experiment, 1.5×10^5 AGS cells were seeded in 6-well (2c.c. medium per well). After 24-hours culture, dilute 110-220 picomoles Scramble/Akt siRNA into 200μl jetPRIME® buffer (referred as a final concentration of 50nM-100nM per well). Then add 4μl of jetPRIME® reagent and incubate for 10 minutes at room temperature. Add the transfection mix to the cells in serum containing medium drop wise. Cells were harvest after 24-36 hours post transfection. For scramble/mTOR/Rictor/Raptor knockdown experiment, 2×10^5 AGS cells were seeded in 6-well (2c.c. medium per well). After 24-hours culture, dilute lipofectamine® RNAiMAX reagent (7.5μl, 9μl, 12μl) and scramble/ mTOR/ Rictor / Raptor siRNA (15, 30, 40 picomoles) into Opti-MEM® medium, respectively. Add

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diluted siRNA into diluted lipofectamine® RNAiMAX reagent and follow the 5 minutes incubation at room temperature. Then add siRNA-lipid complex to cells dropwisely. Cells were harvest after 24-36 hours post transfection.

6. Plasmid DNA transfection

The plasmid DNA was purified by NucleoBond® Xtra Maxi EF (MACHEREY-NAGEL, Germany). 1.2×10^6 AGS cells were seeded in 10 cm-plate overnight. Before transfection, replace the medium freshly (5c.c. per plate). For each 10 cm-plate, dilute 5μg plasmid DNA and 15μl GenJet™ Plus reagent into 250μl serum-free DMEM with high glucose, respectively. Add the diluted GenJet™ Plus reagent immediately to the diluted DNA solution all at once and gently pipette up and down 3-4 times. Incubate the transfection complex for 10 minutes at room temperature and add 500μl GenJet™/DNA mixture drop-wise onto the medium. After 15-18 hours replace the medium by fresh RPMI-1640 supplemented with 10% FBS.

7. Cell sorting

After 36 hours transfection, the transfected cells on 10-cm plate were washed by 1X DPBS and detached by accumax (Innovative Cell Technologies, USA). Cells were collected by 1X sorting buffer with PI and centrifuge at 400×g, 3mins. Discard the

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supernatant and re-suspend cell pellet in appropriate volume of sorting buffer (usually 3-5×10^7 cells were re-suspend in 1 ml sorting buffer). Cells were sorted on FACSAria (BD Bioscience, San Jose, CA) through the service provided by the Flow Cytometric Analyzing and Sorting Core (the First Core Laboratory, National Taiwan University College of Medicine). The sorted cells in RPMI-1640 supplemented with 10% FBS and antibiotics were centrifuge at 400×g, 5mins and wash by RPMI-1640 supplemented with 10% FBS without antibiotics. The cells were seeded in 12-well plate (2×10^5/ per well) overnight. The cell number was counted before *H. pylori* co-culture.

8. Statistics

Statistical analysis of experimental groups was performed by unpaired Student's t-tests. $P<0.05$ was considered significant. (P value $\leqq 0.05$ = *, $\leqq 0.01$ = **, $\leqq 0.001$ = ***, $\leqq$ 0.0001 = ****) The quantification of Western blots was performed by ImageJ.

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Results

I. *H. pylori* enhanced the apoptosis of AGS cells while treat with TRAIL, and phosphorylation of Akt as well as FLIPs expression in AGS cells decreased concurrently.

Previous study has reported that, the apoptosis of AGS cells increased after co-culture with *H. pylori* for 12 hours and treat with TRAIL (40ng/ml) sequentially (Lin et al., 2014). To start with, we confirmed the TRAIL-mediated apoptosis was severely increased during co-culturing the human gastric epithelial cell line (AGS) with *H. pylori*, but couldn't be induced when only incubated with either *H. pylori* or TRAIL (Fig. 1A). Also, the pro-caspase-8 was cleaved into active form only when co-cultured with *H. pylori* and TRAIL engagement simultaneously. This result was consistent with the previous result in our laboratory. Furthermore, the phosphorylation of Akt in both of S^{473} and Thr^{308} site, were decreased while *H. pylori* co-cultured, and this phenomenon was correlated with FLIPs downregulation (Fig. 1B).

Moreover, previous study has shown that *H. pylori*-induced gastric epithelial cell apoptosis is associated with increased of Fas receptor expression (Jones et al., 1999). Besides, TRAIL shows sequence homology to Fas ligand (Wiley et al., 1995). We wondered that whether Fas ligand exerts the same effect as TRAIL while co-cultured with *H. pylori*. However, the enhancement of apoptosis only occurred while treated

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with TRAIL but not Fas ligand. This result highlighted the importance of TRAIL-mediated apoptosis while *H. pylori* infection in AGS cells (Fig. 2).

II. The expression of FLIPs was decreased and enhanced TRAIL-mediated apoptosis in AGS cells after knockdown of Akt.

FLIPs is known to play a crucial role in preventing TRAIL-mediated apoptosis (von Karstedt et al., 2017). Furthermore, our laboratory has reported that *H. pylori* downregulated FLIPs expression, hence increasing TRAIL-sensitivity in AGS cells (Lin et al., 2014). Associated with our observation in the previous section, we wondered that whether Akt is able to regulate FLIPs expression in AGS cells. In order to clarify the correlation between Akt and FLIPs, we transfected Akt siRNA in order to knockdown Akt expression in AGS cells. The result has shown that decreased of Akt expression resulted in FLIPs expression downregulated (Fig. 3A). Besides, loss of Akt also enhanced the TRAIL-mediated apoptosis in AGS cells (Fig. 3B). Our results have demonstrated that Akt enabled to regulate FLIPs expression and prevented TRAIL-mediated apoptosis in AGS cells. Moreover, these data suggested that loss of Akt activity is a possible pathway for *H. pylori* to enhance TRAIL-mediated apoptosis in AGS cells.

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III. Knockout of AIP4 reduced *H. pylori*-induced TRAIL-mediated caspase-8 activation.

Several mechanism has been reported to participate in Akt-FLIPs pathway in TRAIL-mediated apoptosis. One of them has been demonstrated that was modulated by AIP4, an ubiquitin E3 ligase, to stimulate FLIPs degradation through proteasome pathway. Previous data in our laboratory has shown that *H. pylori* infection resulted in activating AIP4 activity (Woan-Yu Lin, 2016). In order to prove the role of AIP4 in *H. pylori*-induced TRAIL-mediated apoptosis, we adopted AIP4 KO AGS cells to examine that whether knockout of AIP4 rescued *H. pylori*-induced TRAIL-mediated apoptosis. Our data has shown that, compared to WT AGS cells, AIP4 KO AGS cells have less active cleavage-caspase8 while *H. pylori* co-cultured and TRAIL treatment (Fig. 4).

Although we have shown that knockout of AIP4 reduced the *H. pylori*-induced TRAIL-mediated caspase-8 activation, we have found that there still remains a certain amounts of active caspase-8 while co-cultured with *H. pylori* and treated with TRAIL. We have sought for another E3 ubiquitin ligase cbl, which has also been reported to stimulate FLIPs degradation in mTOR-dependent but Akt-independent manner (Zhao et al., 2013). We have observed that co-cultured with *H. pylori* caused cbl expression increased (Fig. 5A). In addition, the interaction between cbl and FLIPs increased while

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H. pylori infection. However, knockdown of cbl failed to mitigate *H. pylori*-induced TRAIL-mediated apoptosis (Fig. 5C).

IV. mTOR expression was decreased after *H. pylori* co-cultured.

In order to investigate that how *H. pylori* modulated intracellular Akt activity, we have screened the upstream molecules which control the Akt activity. Among of them, we have found the mTOR expression decreased was correlated with Akt phosphorylation as well as FLIPs expression decreased while co-cultured with *H. pylori* in time-dependent manner (Fig. 6A). Besides, we found that *H. pylori* infection not only resulted in decreasing mTOR expression but also the expression of Rictor and mSin1, which are the other obligate subunit for mTORC2 formation. Moreover, the phosphorylation $mTOR^{S2481}$, an intact marker for mTORC2 activity (Copp et al., 2009), reduced while *H. pylori* infection. These results have shown that *H. pylori* may modulate Akt activity through hindering mTORC2 formation to suppress mTORC2 activity. We have also investigated that how *H. pylori* prompted mTOR expression decreased. We found that *H. pylori* did not affect mTOR gene expression but promote mTOR degradation through proteasome (Fig. 6C, 6D).

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V. Inhibition of mTOR reduced the expression of FLIPs and induced TRAIL-mediated apoptosis in AGS cells.

Since we have observed that mTOR expression decreased was correlated with Akt de-phosphorylation as well as decreased of FLIPs expression. We wondered that whether mTOR enable to regulate FLIPs expression in AGS cells. First, we utilized mTOR inhibitor (Torin1 & Rapamycin) to investigate the relevance between mTOR and FLIPs. We found that while using rapamycin, the mTORC1 inhibitor, caused the slightly decreased of FLIPs expression but the expression of FLIPs was remarkably reduced while treat with Torin1, the pan-mTOR inhibitor (Fig. 7A). Additionally, we pretreated Torin1 to AGS cells and followed the TRAIL treatment afterward also elevated the TRAIL-mediated apoptosis in gastric epithelial cells (Fig. 7B). This result has revealed that pan-mTOR inhibition was able to heighten the TRAIL-mediated apoptosis signaling in gastric epithelial cell via controlling FLIPs expression.

Moreover, in order to exclude the off-target effect while using the mTOR inhibitors, we also transfected mTOR siRNA into AGS cells. Knockdown of mTOR in AGS cells strongly decreased the FLIP expression and enhanced the TRAIL-mediated apoptosis in AGS cells (Fig. 8A, 8B). Since mTOR synergistic with other protein to form distinct protein complexes, known as mTOR Complex 1 (mTORC1) and 2 (mTORC2) (Saxton and Sabatini, 2017), we further discriminated the role of mTORC1/mTORC2 in

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TRAIL-mediated apoptosis signaling. Knockdown of Raptor and Rictor, which is the core protein of mTORC1 and mTORC2, to interfere the mTORC1/2 activity. While loss of mTORC1 activity, the expression of FLIPs slightly reduced. However, while loss of mTORC2 activity, the expression of FLIPs dramatically decreased (Fig. 8A). These data demonstrated that mTOR, especially mTORC2, was able to regulate FLIPs expression. In addition, mTOR provided resistance to TRAIL-mediated apoptosis in AGS cells. Above all, these result implied that *H. pylori* may break the TRAIL-resistance in AGS cells through inhibiting mTOR activity.

VI. Enhancement of mTOR-Akt signaling pathway reduced *H. pylori*-induced TRAIL-mediated apoptosis signaling.

The data we performed have already shown that mTORC2 lost its function when co-cultured with *H. pylori*. Since mTORC2 is the primary kinase for activating Akt, we suspected the functional loss of mTORC2 resulted in the Akt de-phosphorylation while *H. pylori* infection. First of all, in order to elevate the mTORC2 activity, we co-overexpressed the peYFP-C1-mTOR/pRK5-HA-YFP-Rictor plasmid to enforce more mTORC2 complex formation in gastric epithelial cells. We sorted out the GFP^+ cells and found the phosphorylation of Akt^{S473}, the downstream substrate of mTORC2 (Sarbassov et al., 2005), was increased compared to the vector group (peGFP-C1, GFP^+ cells) (Fig. 9A, 9B). By taking advantage of this strategy, we found that while enhancing

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the mTORC2 activity, the *H. pylori*-induced TRAIL-mediated apoptosis was attenuated in gastric epithelial cells (Fig. 10A). Furthermore, we found that augmentation the mTORC2 activity sustained the Akt phosphorylation level while *H. pylori* infection (Fig. 10B). These result demonstrated that mTORC2 was crucial for AGS cells to resist with TRAIL-induced apoptosis while *H. pylori* infection through sustaining the phosphorylation of Akt.

VII. *H. pylori*-induced autophagy could be attenuated while enhanced mTOR-Akt signaling pathway in AGS cells.

Reduced Akt phosphorylation also inhibits downstream mTORC1 signaling pathway (Saxton and Sabatini, 2017). In the previous result, we have displayed that *H. pylori* decreased mTOR expression as well as reduced Akt phosphorylation (Fig 1B, 6A, 6B). These data implied that *H. pylori* may also suppress mTORC1 downstream signaling pathway. We have displayed that loss of mTORC1 activity also resulted in FLIPs decreased, although it was not severed as strong as the effect of losing mTORC2. mTORC1 is also known for controlling protein turnover through autophagy. Besides, there has been reported that inhibition of mTOR with activating autophagy flux downregulates c-FLIP levels and contribute toward the enhancement of TRAIL-sensitivity (Xu et al., 2016; Zhang et al., 2014). Combined with the data of decreased mTOR expression while infection with *H. pylori* (Fig. 6A), these data prompted us to

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investigate about whether *H. pylori*-induced autophagy contributes to enhancement of TRAIL-mediated apoptosis in AGS cells. Indeed, we found *H. pylori* infection induced cellular autophagy (Fig. 11) by elevating the expression of LC3-II, an autophagy marker. In order to examine whether mTOR inhibition was sufficient to induce autophagy, we utilized PP242, the pan-mTOR inhibitor, to observe the effect of mTOR inhibition-mediated autophagy in AGS cell lines. We found the expression of LC3-II has increased as a dose-dependent manner. Besides, LC3-II expression increased was correlated with FLIPs downregulation (Fig. 12A). We excluded the effect of mTORC2 by treating with Metformin, an autophagy inducer, to address the relevance between autophagy and FLIPs expression. We have also found that FLIPs expression reduced while activating the autophagy flux (Fig. 12B). Besides, treating with Metformin also enhanced TRAIL-mediated apoptosis (Fig. 12C). We further examined that whether the autophagy flux could be turn off by enhancing the mTORC1 activity through overexpressing mTOR/Rictor or Akt plasmid in gastric epithelial cells. We have found that enhancing the mTORC2-Akt axis or Akt only successfully attenuated the autophagy process (Fig. 13A, 13B). Taken together, we have found that attenuation of autophagy process was correlated with reducing *H. pylori*-induced TRAIL-mediated apoptosis (Fig. 13, Fig. 10).These data suggested that autophagy may also contribute in *H. pylori*-induced TRAIL-mediated apoptosis.

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Discussion

I. *H. pylori*-induced TRAIL-mediated apoptosis through inhibiting the intracellular mTOR-Akt signaling pathway.

Based on the previous founding in our laboratory, Akt plays a crucial role in *H. pylori*-induced TRAIL-mediated apoptosis. We wondered that how does *H. pylori* reduce Akt phosphorylation state. In this study, we have found the expression of mTOR, the core protein of mTORC1/2, decreased as *H. pylori* co-cultured. Besides, we found the S2481 phosphorylation site of mTOR, which is an intact mTORC2 activity marker also decreased (Copp et al., 2009). This data indicated that mTORC2 complex activity was disrupted while *H. pylori* co-cultured. However, we haven't investigated the details about other obligate subunit such as the phosphorylation of mSin1, which has been reported to associate with the positive feedback loop between Akt and mTORC2 (Yang et al., 2015). It is crucial for us to further understand the *H. pylori*-modulated mTORC2-Akt axis in order to realize the whole picture of *H. pylori*-induced TRAIL-mediated apoptosis signaling.

Besides, we have developed a system to elevate the activity of mTORC2 by co-overexpressing plasmids with mTOR/Rictor. At first, we tried to enhance the pan-mTOR signaling pathway through overexpressing mTOR plasmid only. However, we

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found the phosphorylation of Akt^{S473} site decreased after overexpressed mTOR plasmid. That may be the negative feedback exerted by mTORC1, which has been reported to negatively regulate receptor tyrosine kinases (RTKs) signaling (Hsu et al., 2011; Yu et al., 2011). Since mTORC2 is the upstream molecule of Akt, we turned to consider enhancing mTORC2 activity to upregulate mTOR-Akt signaling pathway. We have consulted several papers which also attempt to enhance mTORC2 activity by overexpressing the plasmid of Rictor (Laugier et al., 2015; Masri et al., 2007). In line with previous reports, we found that overexpression of rictor in AGS cell lines indeed enhanced the Akt phosphorylation on S473 site. Furthermore, we got the largest activation of Akt while co-overexpressing mTOR/Rictor plasmid simultaneously. Therefore, we adopted this method to conduct the afterward experiment. But still, this model existed some unresolved question: Does the mTOR/Rictor protein we have overexpressed form the functional mTORC2 indeed? Although we have monitored the mTORC2 activity by direct downstream substrate Akt, it may still remain other unidentified pathway to enhance the Akt activity since the network signaling of mTOR is complicated. To further clarify this question, we should observer whether there is more interaction between mTOR and Rictor by co-immunoprecipitation while co-overexpressing mTOR/Rictor plasmid.

As described previously, we found the negative impact on Akt^{S473} phosphorylation

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while overexpressing mTOR only. We got the largest Akt activation in our hands when co-overexpressed mTOR and Rictor plasmid, despite the Akt^{S473} phosphorylation does not reach as same intensity as directly overexpressed Akt compared to previous result. There may be two reasons: one is that the physiological regulation of mTORC2 toward Akt is limited; another explanation is that our sorting strategy is imprecise (Fig. 9A). We simply sorted out the GFP^+ cells while co-overexpressing peYFP-C1-mTOR and pRK5-HA-YFP-Rictor, however, we couldn't identify every single cell expressing YFP^+ was homogeneously expressing mTOR and Rictor at same ratio since the mTOR/Rictor protein expressed the same fluorescent protein. The possible solution is to construct different fluorescent protein in one of these plasmids, and clarify the appropriate ratio for mTOR: Rictor while enhancing the activity of mTORC2.

Besides, the previous study in our laboratory has shown that *H. pylori* also dephosphorylated PTEN. The phosphorylated PTEN is proved to be less active (Song et al., 2012). Thus, it implied that downregulation of Akt phosphorylation may also be regulated by more active PTEN while *H. pylori* infection. We have conducted transient knockdown of PTEN in AGS cell lines and followed the *H. pylori* co-cultured and TRAIL treatment. The data showed that while PTEN knockdown, the *H. pylori*-induced TRAIL-mediated apoptosis was mitigated (Szu-Ying, Chen. 2017). Yet it raised a question: Whether PTEN or mTORC2-mediated pathway is dominant in *H. pylori*-

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induced TRAIL-mediated apoptosis? Previous perspective may take PTEN and mTORC2 as two independent events in regulation of Akt. However, there are increasing evidence shows that PTEN also regulates mTORC2 directly or indirectly. Bhattacharya et al., have observed that PTEN negatively regulates mTORC2 formation by promoting Rictor phosphorylation (Thr1135), which is known to inhibit mTORC2 activity (Bhattacharya et al., 2016; Julien et al., 2010). Thus, PTEN de-phosphorylation while *H. pylori* infection may also contributes to the inhibition of mTORC2 activity (Fig. 6B). Besides, knockdown of PTEN also gives rise to hyperactive mTORC2 activity (Bhattacharya et al., 2016), hence rescuing *H. pylori*-induced TRAIL-mediated apoptosis couldn't take it as a PTEN-dependent effect only. Also, PTEN counteracts PI3K to dephosphorylate PIP_3 (Song et al., 2012), Liu et al., have also observed that the PH domain of mSin1 binds to the mTOR kinase domain in the absence of PIP_3, thus suppressing the mTOR kinase activity. Overall, loss of mTORC2 activity may also by reason of *H. pylori*-induced PTEN activation.

II. *H. pylori* downregulated FLIPs expression through reducing Akt phosphorylation.

Previous study in our laboratory has shown that *H. pylori* increased the activity of AIP4 to ubiquitinate FLIPs through downregulating Akt phosphorylation. In this study,

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we have further examined the role of AIP4 in *H. pylori*-induced TRAIL-mediated apoptosis signaling. By taking advantage of AIP4 KO AGS cells, we have found that *H. pylori*-induced TRAIL-mediated caspase-8 activation was mitigated while compared to WT AGS cells. However, the AIP4 KO AGS cells did not fully block the *H. pylori*-induced TRAIL-mediated apoptosis signaling (Fig. 4). This data suggested that there may have other mechanisms which participated in $FLIP_s$ downregulation by *H. pylori*. We have knockdown the Akt expression in AGS cells and found the FLIPs expression was strikingly decreased (Fig. 3A). This result implicated that Akt activity is crucial for regulating FLIPs expression. There are abundant evidences for displaying the ability of Akt to regulate FLIPs but several of them elucidate the mechanisms in details (Chen et al., 2001; Nam et al., 2003; Panka et al., 2001). Akt participates in controlling translation process, post-translational modification and autophagy, and those aspect of Akt have been reported to affect FLIPs expression (Panner et al., 2009; Panner et al., 2005; Zhang et al., 2014). In this section, the different aspect of controlling FLIPs expression by Akt would be discussed.

(a.) Translation

Akt is known to master the translation process through modulating mTORC1 activity. In brief, PI3k-mediated activation of Akt leads to phosphorylation of TSC2, which results in inhibition of the TSC1/2 complex. In the absence of TSC1/2, the small

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GTPase Rheb is able to enhance mTOR activity and stimulate activation of downstream targets of mTOR, the 70-kDa ribosomal S6 kinase 1 (S6K1) and the eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4EBP1). Both of S6K1 and 4EBP1 are master regulator of cellular translation process. Besides, Panner et al., have shown that mTOR controls FLIPs translation and TRAIL sensitivity in glioblastoma multiforme cells. Since *H. pylori* decreased mTOR expression and dephosphorylated Akt sequentially (Fig. 1B, Fig. 6), we suspected *H. pylori* may also affect FLIPs expression through mRNA level. However, we simply measured the FLIPs mRNA level with/without *H. pylori* infection and found that there was no significant difference. This data revealed that *H. pylori* did not affect the number of FLIPs mRNA, but it still can't exclude the possibility of *H. pylori* interfered the FLIPs translation process. As described by Panner et al., they have found the ribosomal distribution of FLIPs mRNA is critical for different GBM cell lines to exert TRAIL-resistance (Panner et al., 2005). Whether *H. pylori* modulates the distribution of FLIPs mRNA in non-translating monoribosomes or translating polyribosomes remains an interesting question to explore.

(b.) Post-translational modification

So far, the well-characterized pathway for the FLIPs regulation by Akt till now is through modulating AIP4 activity (Panner et al., 2010). In this study, we have further demonstrated that AIP4 participated in the *H. pylori*-induced TRAIL-mediated

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apoptosis signaling. However, the *H. pylori*-induced TRAIL-mediated apoptosis did not completely mitigate even if we knockouted AIP4 in AGS cells. Thus, we suspected that there may have other undefined mechanisms which was regulated by Akt also participate in *H. pylori*-induced TRAIL-mediated apoptosis signaling.

(c.) Autophagy

Akt de-phosphorylation inhibits the downstream mTORC1 signaling pathway. In this study, we also found *H. pylori* infection induced cellular autophagy (Fig. 11). We have found that enhancing the mTORC2-Akt axis or Akt only successfully attenuated the autophagy process. We have shown that attenuation of autophagy process correlated with reducing *H. pylori* induced TRAIL-mediated apoptosis, suggesting that autophagy may contribute in *H. pylori*-induced TRAIL-mediated apoptosis. However, both methods we have utilized in this study (Overexpressed mTORC2/Akt) can't solely observe the effect of autophagy process on *H. pylori* induced TRAIL-mediated apoptosis. To further resolve the impact of autophagy on *H. pylori*-induced TRAIL-mediated apoptosis, knockdown of Atg5 or LC3 is a common technique to block the autophagy process. Although we have demonstrated the importance of mTOR in *H. pylori*-induced TRAIL-mediated apoptosis, the detail mechanisms inside are still worth to investigate.

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III. *H. pylori* downregulated FLIPs expression through Akt-independent mechanisms.

There is a report that demonstrated mTORC2 is involved in regulation of FLIPs degradation and sensitivity of TRAIL-induced apoptosis through c-cbl, an E3 ubiquitin ligase (Zhao et al., 2013). Zhao et al., have demonstrated that PP242, an ATP-competitive inhibitors of mTOR, cooperates with TRAIL to enhance apoptosis in NSCLC cell lines. Besides, they identified the mTORC2 inhibition mediates cbl to promote FLIPs degradation. Surprisingly, the author found that FLIPs downregulation by PP242 could not be restored by constitutively active form of Akt. This paper implicates that cbl-mediated FLIP degradation is a mTORC2-dependent, but Akt-independent mechanism. We have also explored the possibility of cbl involved in *H. pylori*-mediated TRAIL-mediated apoptosis, since AIP4 KO AGS cells couldn't fully block the activation of caspase-8 while *H. pylori* sensitizes TRAIL-mediated apoptosis in AGS cells. However, we failed to rescue the *H. pylori*-mediated TRAIL-mediated apoptosis by knockdown of cbl (Fig. 5). Although we have detected more interaction between cbl and FLIP, cbl may have other physiological role on FLIPs but not mediating FLIPs degradation in *H. pylori*-induced TRAIL-mediated apoptosis. These data further support that mTOR-Akt-FLIP axis is dominant in *H. pylori*-induced TRAIL apoptosis signaling.

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There is another report shows that c-FLIP is a target of deltex1, an E3 ubiquitin ligase, in gastric cancer, although they focus on studying the $FLIP_L$ expression. Their data also show that overexpressed Deltex1 decreases both $FLIP_L$/$FLIP_s$ expression in AGS cell lines. Finally, they found Deltex1-mediated $FLIP_L$ degradation is through the endosome-lysosomal pathway but not proteasome-independent degradation (Hsu et al., 2018). Although our data have shown that *H. pylori*-mediated FLIP degradation is mainly through proteasome (Fig. 6D), the possibility of deltex1 to participate in *H. pylori*-mediated TRAIL-mediated apoptosis signaling is still another direction to investigate.

IV. The upstream regulation of mTORC2 activity and the possible pathway for *H. pylori* to disrupt the mTORC2 activity in AGS cell lines.

The upstream regulation of mTORC2 is not well-defined, although primarily functions as an effector of insulin/PI3K signaling. Besides, the ribosome association is also known for being mTORC2 activator (Kim et al., 2017; Sarbassov et al., 2005; Saxton and Sabatini, 2017; Zinzalla et al., 2011). Despite a lots of unknown remains on regulating mTORC2 activity, there are increasing evidence which have demonstrated that the localization is important for mTORC2 activation. Liu et al., have also observed the PH domain of mSin1 which binds to the mTOR kinase domain suppresses the

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mTOR kinase activity in the absence of PIP_3. However, while PIP_3 and mTORC2 both localized on the membrane, PIP_3 relieves inhibition of mTOR, leading to Akt phosphorylation (Liu et al., 2015). Besides, Ebner et al., have exerted an mTORC2 reporter to well-identify the localization of mTORC2 is mainly on plasma membrane and outer mitochondrial membrane, coupled with a subpopulation on endosomal vesicles. Surprisingly, they have found the mTORC2 present on the plasma membrane is catalytically active and PI3K-independent subpopulation. This argumentation is conflict with Liu et al.,. Conversely, the mTORC2 at early and late endosomes was dependent on the presence of functional PI3K (Ebner et al., 2017). Therefore, it is important to identify which subpopulation of mTORC2 is interfered by *H. pylori* in order to uncover the detail mechanisms of how *H. pylori* sensitized AGS cells to TRAIL-mediated apoptosis.

Another report has displayed that Syndecan-4, a transmembrane heparan sulfate proteoglycan which is synergistic with growth factor receptor, regulates subcellular localization of mTOR complex2 and Akt activation in a PKCα-dependent manner in endothelial cells. Disruption of syndecan-4 is associated with decreased mTORC2 activity by dysregulating subcellular localization of mTORC2 components with the decreased presence of rictor, mLST8 and mSin1 in the rafts. As consequence, Akt activation decreased while syndecan-4 lost its function (Partovian et al., 2008). Besides,

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H. pylori is proved to induce syndecan-4 expression in gastric epithelial cells, which depends on intact cagPAI of *H.pylori* (Magalhaes et al., 2009). We have also demonstrated that *H. pylori*-induced TRAIL-mediated apoptosis depended on intact cagPAI of *H. pylori* (Lin et al., 2014). Moreover, the previous data in our laboratory have revealed that cell surface carbohydrates may involve in *H. pylori*-induced TRAIL-mediated apoptosis. We have found that additionally added heparin sulfate inhibited TRAIL-induced apoptosis by *H. pylori*, but these extra-heparin sulfate slightly affected the binding of *H. pylori* to gastric epithelial cell lines (Ya-chi Yang, 2007). Those associations provide another possibility for investigating how *H. pylori* dysregulated mTORC2 activity.

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Conclusion

In this study, we elucidated about the *H. pylori* modulated TRAIL-mediated apoptosis signaling through downregulating Akt-AIP4-FLIPs pathway. Based on this finding, we have further explored that how *H. pylori* modulates Akt phosphorylation. We have found that *H. pylori* decreased mTOR expression and impeded Akt phosphorylation process sequentially, thus conferring susceptibility to TRAIL-mediated apoptosis.

Taken together, this study strengths that mTOR-Akt signaling pathway is vital for *H. pylori*-induced TRAIL apoptosis signaling and sheds light for therapeutic target in *H. pylori*-associated gastric disease.

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Figures

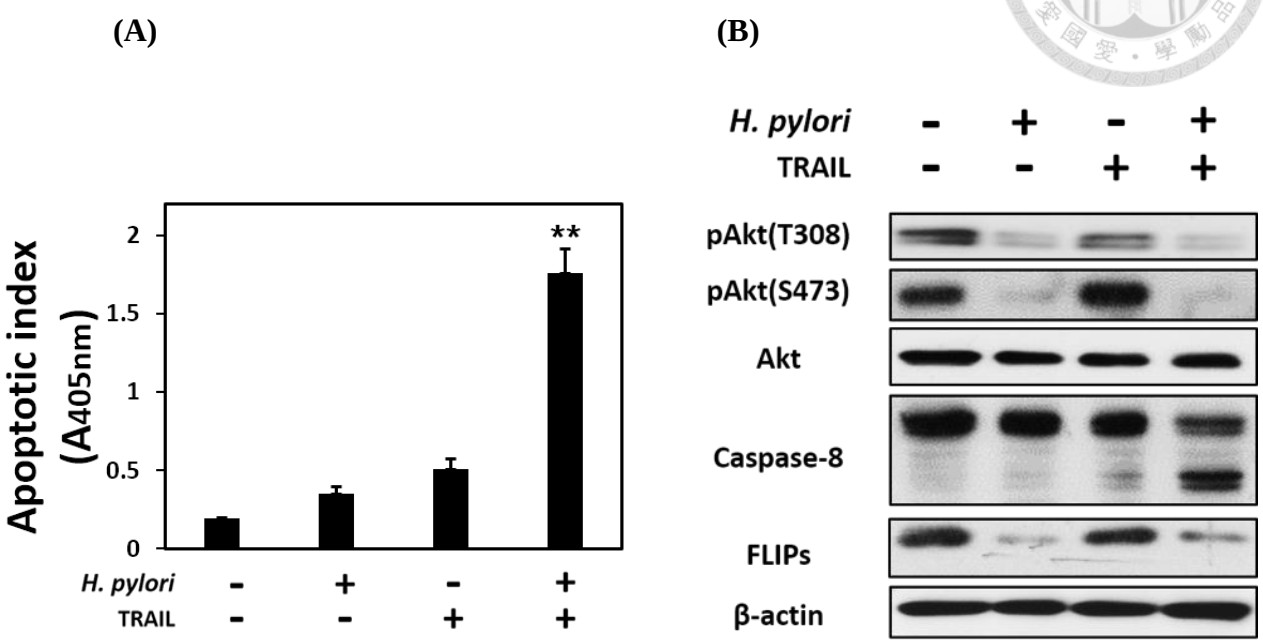

Figure 1. *H. pylori* sensitized AGS cell to TRAIL-mediated apoptosis, and enhancement of TRAIL-mediated apoptosis was correlated with Akt de-phosphorylation and FLIPs downregulation.

Co-cultured AGS cells with *H. pylori* (MOI=100) for 12hrs and treated with TRAIL (40ng/ml) for 3 hours.

(A) The cell apoptotic index was measured by cell death ELISA.

(B) The signaling changes (Akt^{T308}, Akt^{S473}, Caspase-8 and FLIPs) were analyzed by Western blot.

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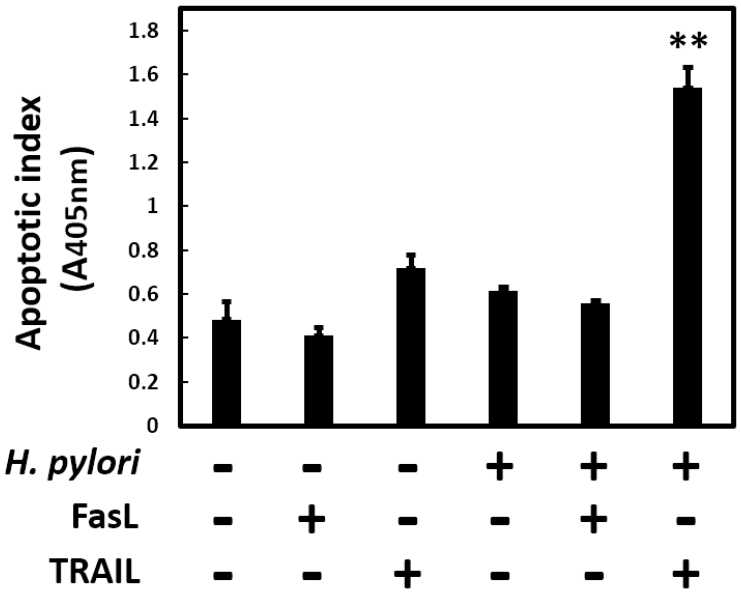

Figure 2. *H. pylori* induced TRAIL-mediated apoptosis but not Fas ligand-mediated apoptosis in AGS cells.

Co-cultured AGS cells with *H. pylori* (MOI=100) for 12 hours and treated with TRAIL (40ng/ml) or Fas ligand (40ng/ml) for 3 hours. The cell apoptotic index was measured by cell death ELISA.

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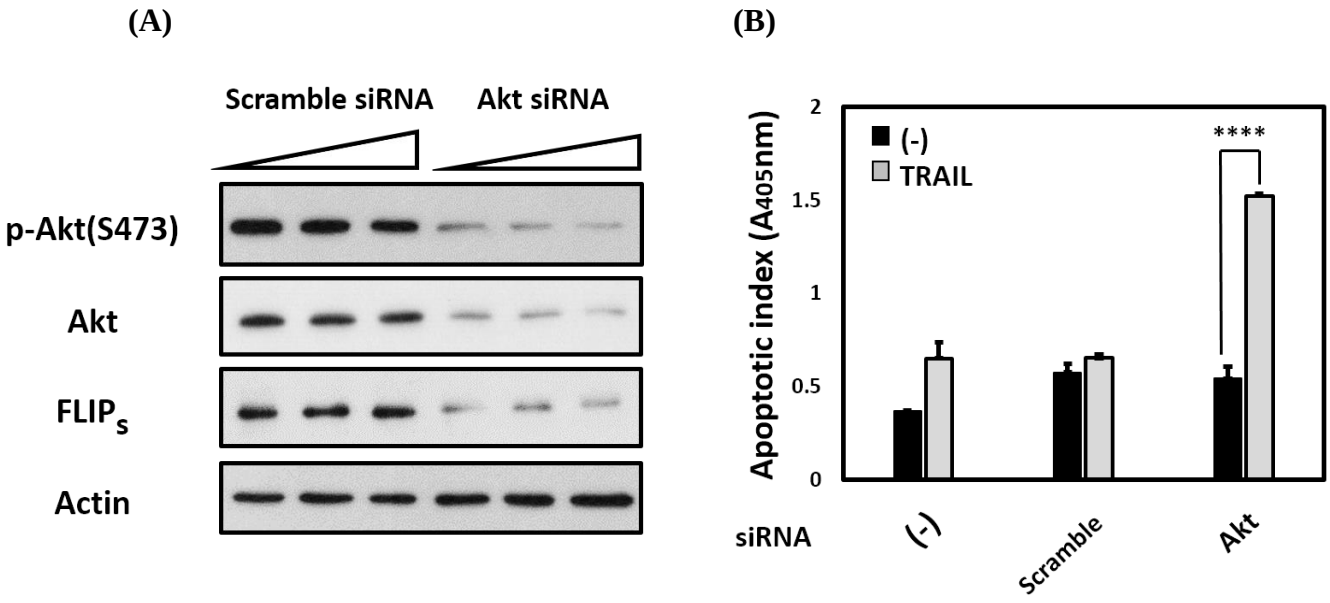

Figure 3. Knockdown of Akt downregulated FLIPs expression as well as enhanced TRAIL-sensitivity in AGS cells

The Akt-siRNA/Scramble siRNA was transfected into AGS cells and harvested the cells after 36 hours.

(A) The signaling change (Akt^{S473}, Akt and FLIPs) was analyzed by Western blot.

(B) The apoptotic index was measured by cell death ELISA following the treatment of TRAIL (40ng/ml) for 3 hours after 36 hours post transfection.

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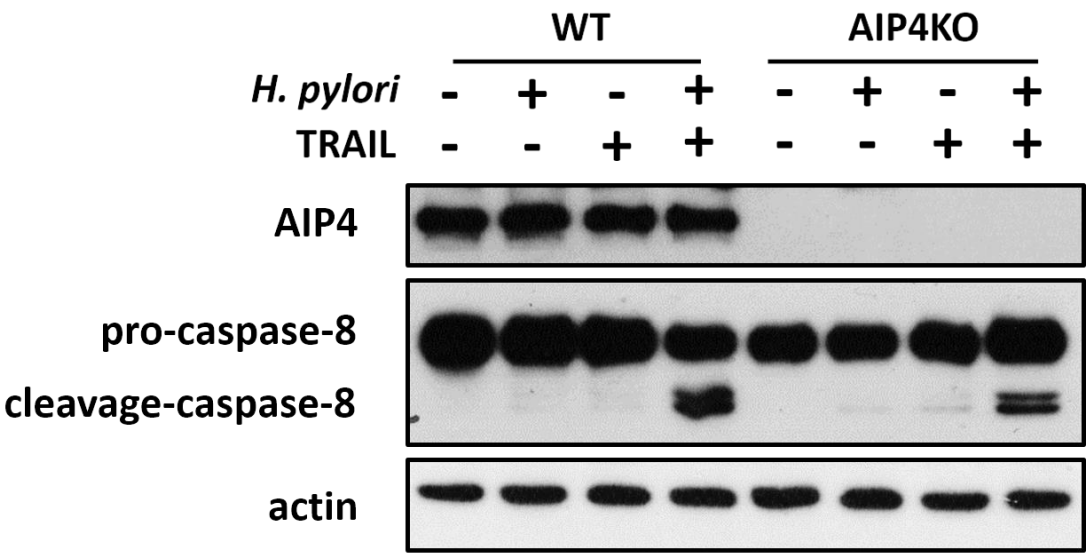

Figure 4. AIP4 knockout reduced the activation of caspase-8 while co-cultured *H. pylori* and TRAIL engagement.

Co-cultured AGS cells with *H. pylori* (MOI=100) for 12 hours and treated with TRAIL (40ng/ml) for 3 hours. The amount of active Caspase-8 was analyzed by Western blot.

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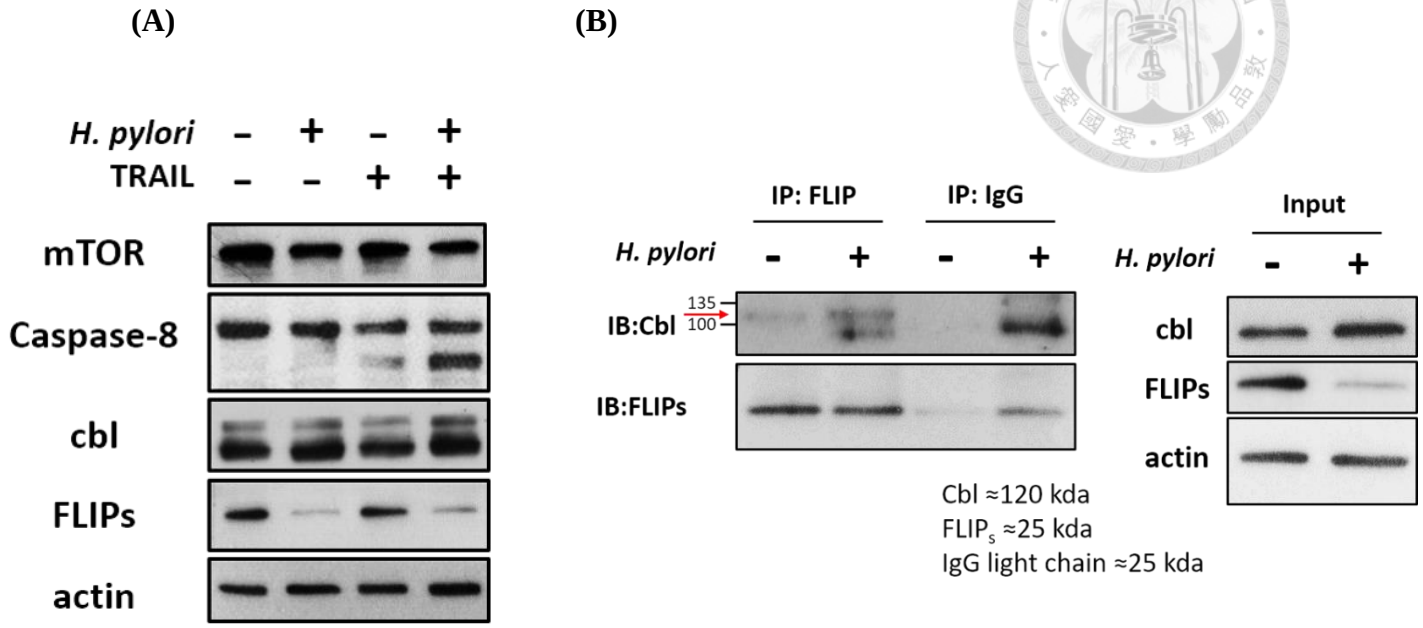

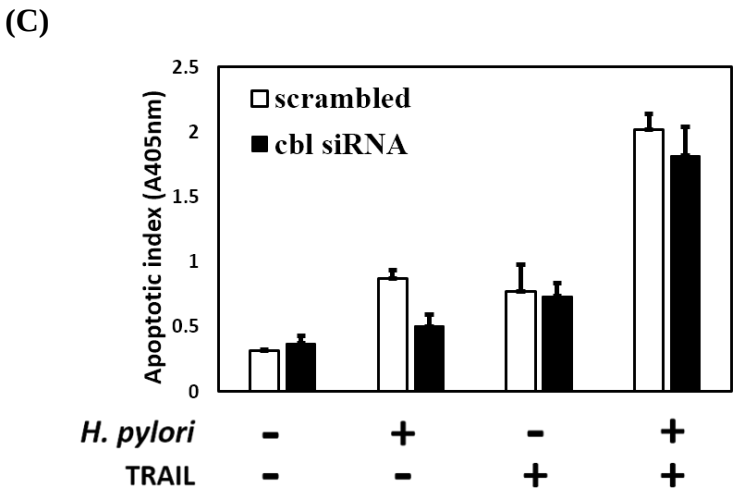

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Figure 5. *H. pylori* increased c-cbl expression and interaction between cbl and FLIPs, but knockdown of cbl did not inhibit *H. pylori*-induced TRAIL-mediated apoptosis.

(A) Co-cultured AGS cells with *H. pylori* (MOI=100) for 12 hours and treated with TRAIL (40ng/ml) for 3 hours. The expression of active Caspase-8, cbl, mTOR, and FLIPs are analyzed by Western blot.

(B) Co-cultured AGS cells with *H. pylori* (MOI=100) for 12 hours and harvested the cell lysate for FLIPs immuno-precipitate, the expression of cbl was analyzed by western blot.

(C) After knockdown cbl for 36 hours, co-cultured cbl-knockdown AGS cells with *H. pylori* (MOI=100) for 12 hours and following the treatment of TRAIL (40ng/ml) for 3 hours. The apoptotic index was measured by cell death ELISA.

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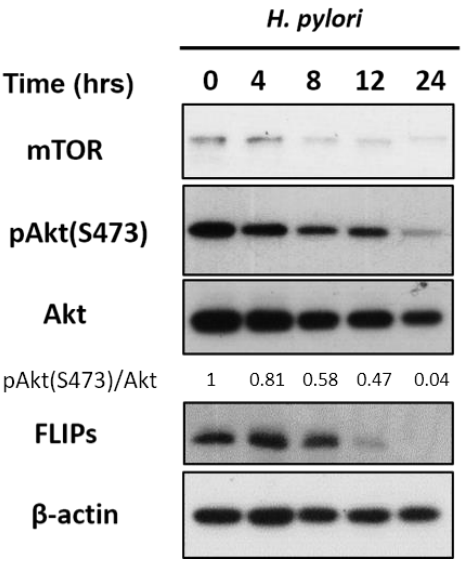

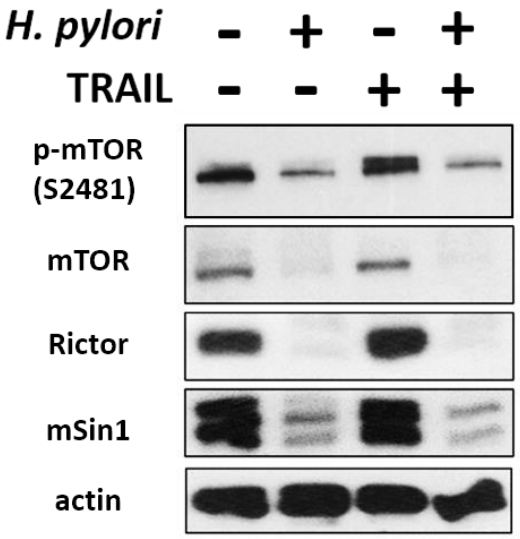

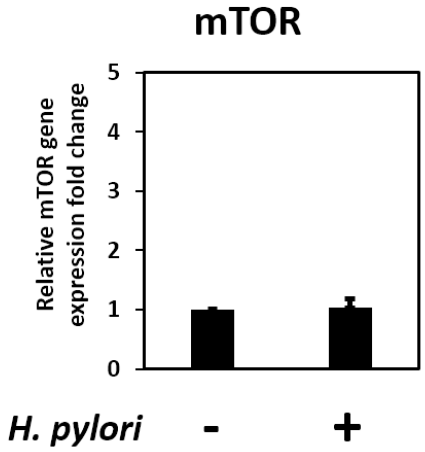

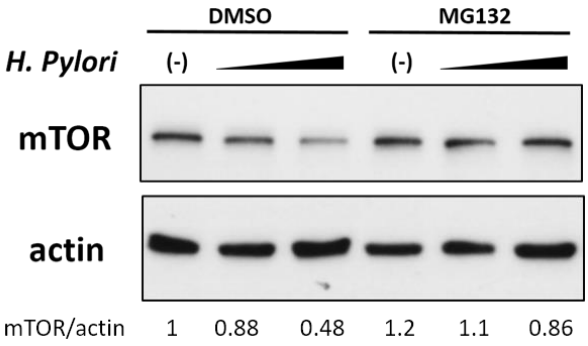

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Figure 6. *H. pylori* reduced the expression of mTOR and related subunit of mTOR complex.

(A) Co-cultured AGS cells with *H. pylori* (MOI=100) following indicated time and harvested the cells. The signaling changes (mTOR, Akt^{S473}, Akt and FLIPs) were analyzed by Western blot.

(B) (C)Co-cultured AGS cells with *H. pylori* (MOI=100) for 12 hours and harvested the cells. The signaling changes (p-$mTOR^{S2481}$, mTOR, Rictor, mSin1) were analyzed by Western blot. (C) The mTOR mRNA level was analyzed by RT-PCR.

(D) The AGS cells were pretreated 1μM MG132 for 1 hour and following the *H. pylori* co-cultured for 12 hours, the signaling expression changes (mTOR, FLIPs) were detected by Western blot.

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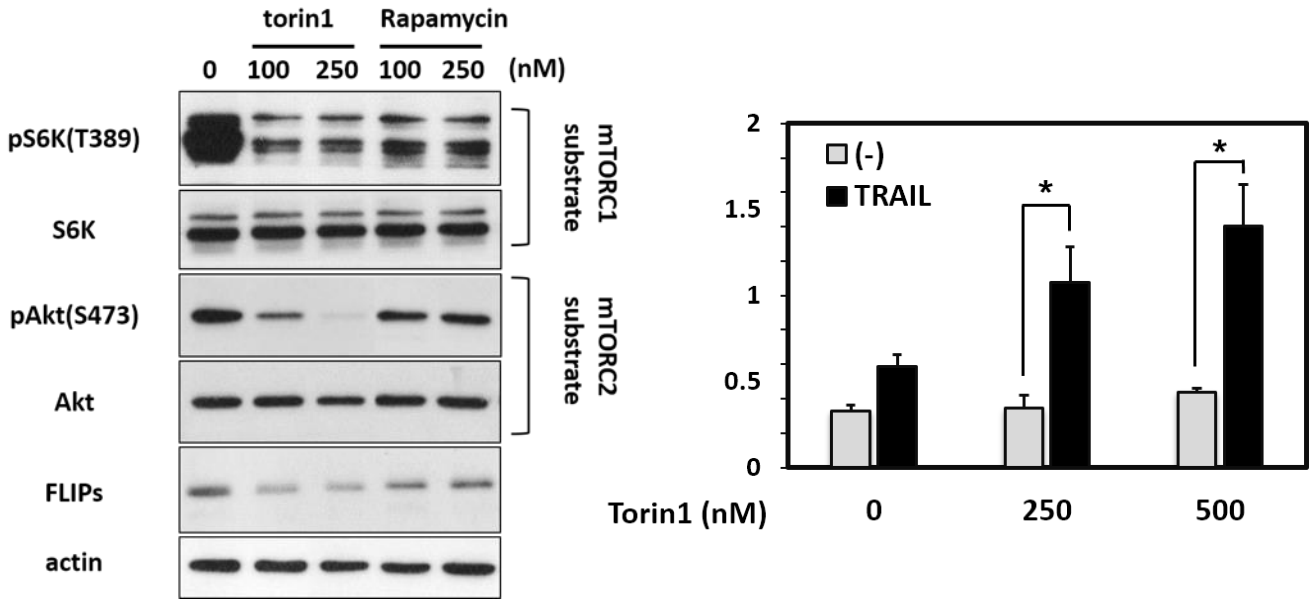

Figure 7. Treatment of mTOR inhibitor decreased the expression of FLIPs and enhanced TRAIL-sensitivity in AGS cells.

(A) The AGS cells were treated with different dose of mTOR inhibitor (Torin1, Rapamycin) for 4 hours and analyzed the signaling changes by Western blot.

(B) The AGS cells were pretreated with Torin1 in different dose for 1 hour and following the TRAIL treatment (40ng/ml) for 3 hours. The apoptotic index was measured by cell death ELISA kit.

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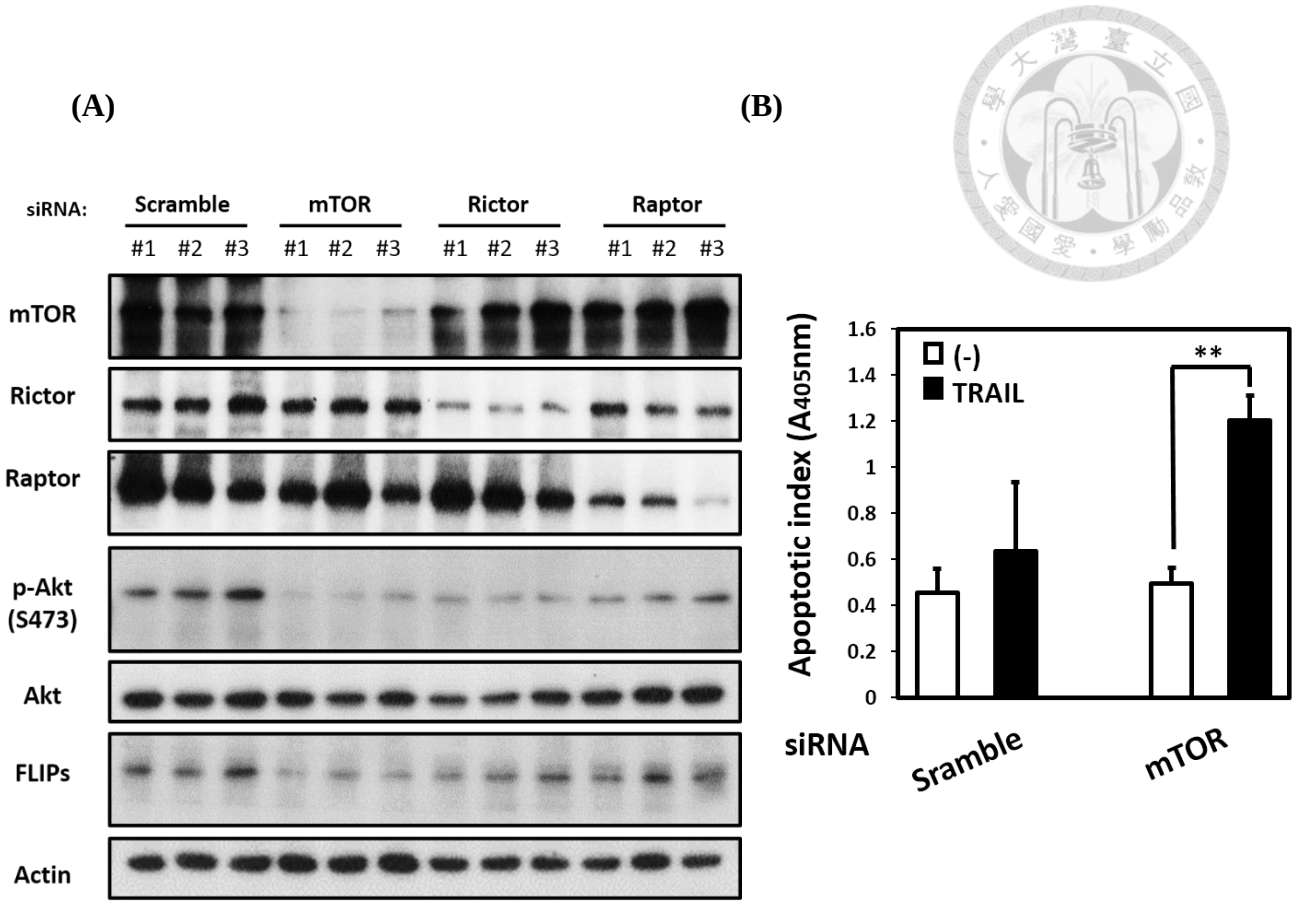

Figure 8. Knockdown of mTOR, Rictor and Raptor downregulated FLIPs expression, and mTOR-knockdown increased TRAIL-mediated apoptosis in AGS cells.

The mTOR/Raptor/Rictor/Scrambled-siRNA was transfected into AGS cells

(A) Harvested the cells after 36 hours. The signaling changes (mTOR, Rictor, Raptor, Akt^{S473}, Akt and FLIPs) were analyzed by Western blot.

(B) After transfected mTOR/Scrambled-siRNA for 36 hours, treated with TRAIL (40ng/ml) for 3 hours. The apoptotic index was measured by cell death ELISA kit.

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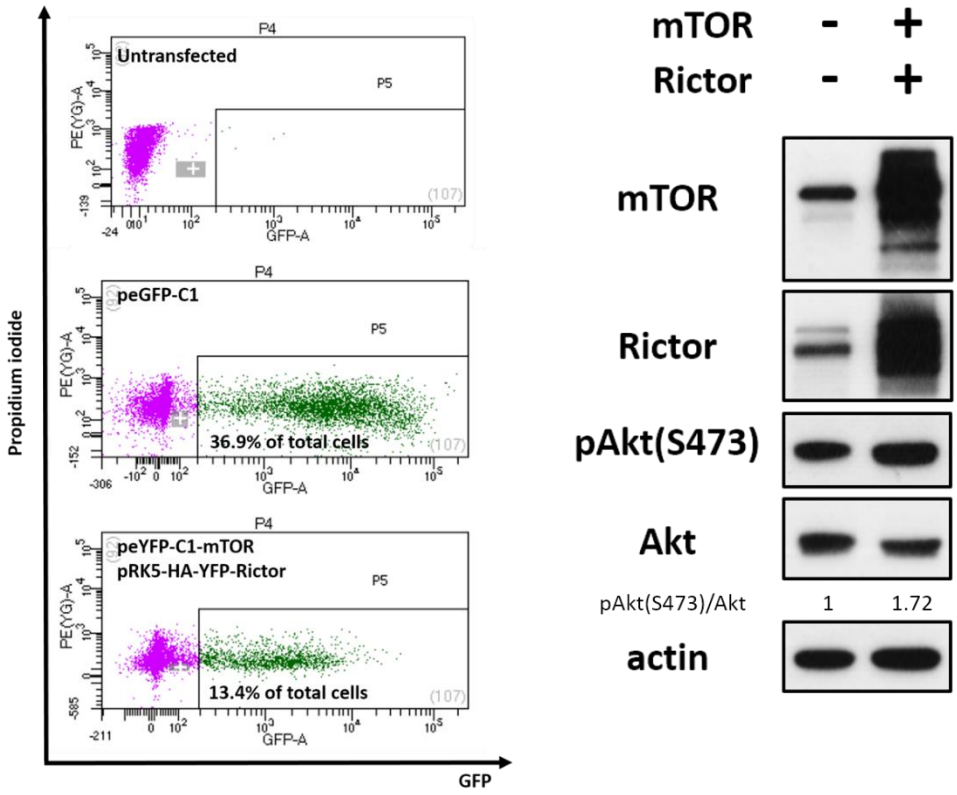

Figure 9. Enhanced Akt^{S473} phosphorylation through co-overexpressing mTOR and Rictor plasmid into AGS cells.

(A)(B) AGS cells were co-overexpressed the mTOR-YFP and Rictor-YFP plasmid for 36hrs and sorted for YFP^{+} cells. The signaling expression changes (mTOR, Rictor, Akt^{S473}, Akt) were detected by Western blot.

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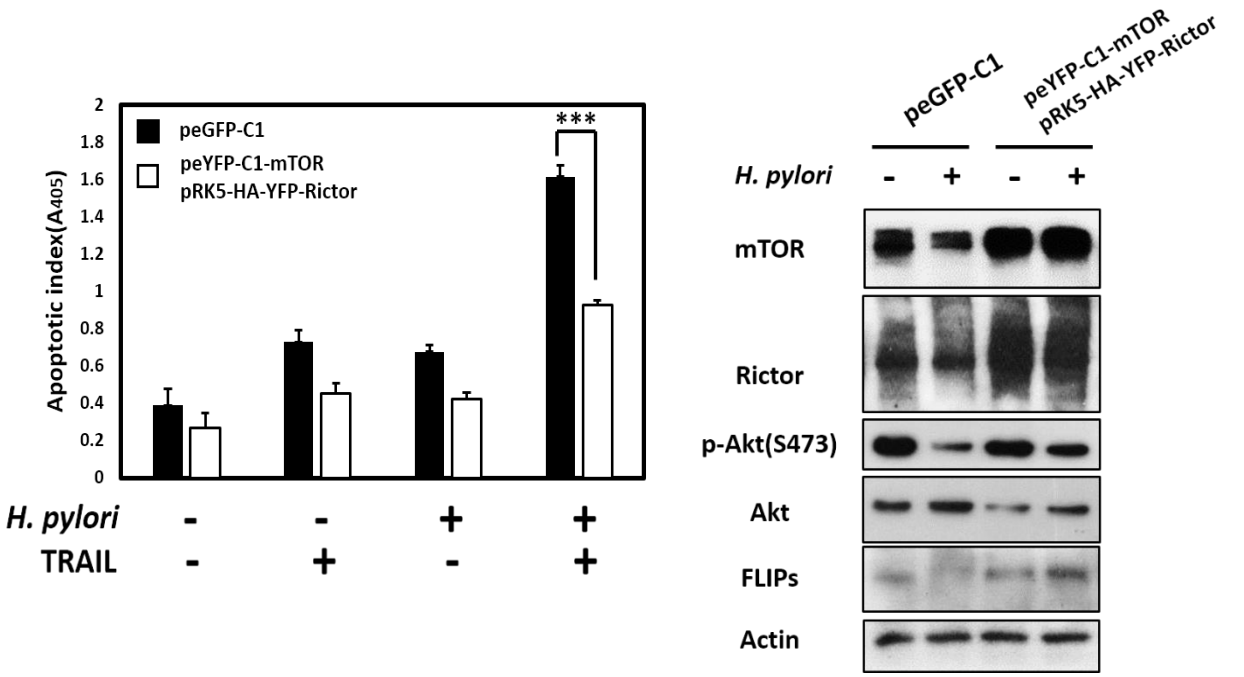

Fig 10. Overexpressed mTOR and Rictor plasmid into AGS cells attenuated *H. pylori*-induced TRAIL-mediate apoptosis signaling.

(A)(B)The cells were overexpressed the indicated plasmid for 36 hours and sorted our GFP^+ /YFP^+ cells. After 24 hours, co-cultured with *H. pylori* for 12 hours, following the TRAIL treatment (40ng/ml) for 3 hours. The apoptotic index was measured by cell death ELISA, and the signaling expression changes were analyzed by Western blot.

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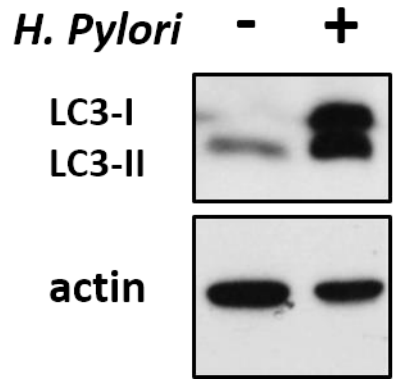

Figure 11. *H. pylori* induced autophagy in AGS cells.

Co-cultured AGS cells with *H. pylori* (MOI=100) for 12 hours and harvested the cells.

The amount of LC3-I/ LC3-II was analyzed by Western blot.

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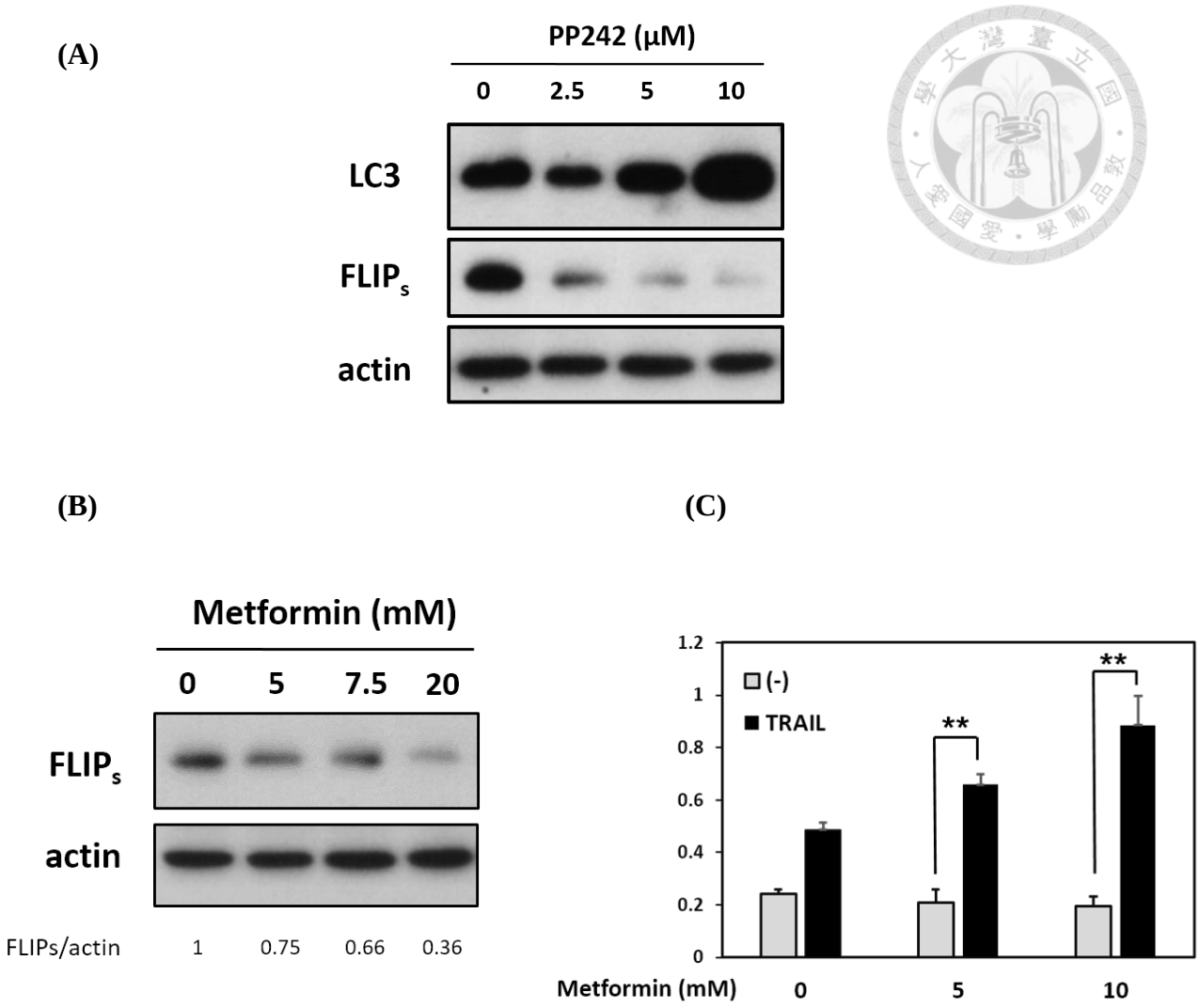

Figure 12. Inhibition of mTOR resulted in autophagy and associated with FLIPs downregulation.

(A) The AGS cells were treated with PP242 or (B)(C) Metformin in indicated concentration for 12 hours and (A)(B) harvested the cells to observe the signaling expression changes by Western blot. (C) Treated with metformin for 12 hours and followed the TRAIL treatment (40ng/ml) for 3 hours. The apoptotic index was measured by cell death ELISA kit.

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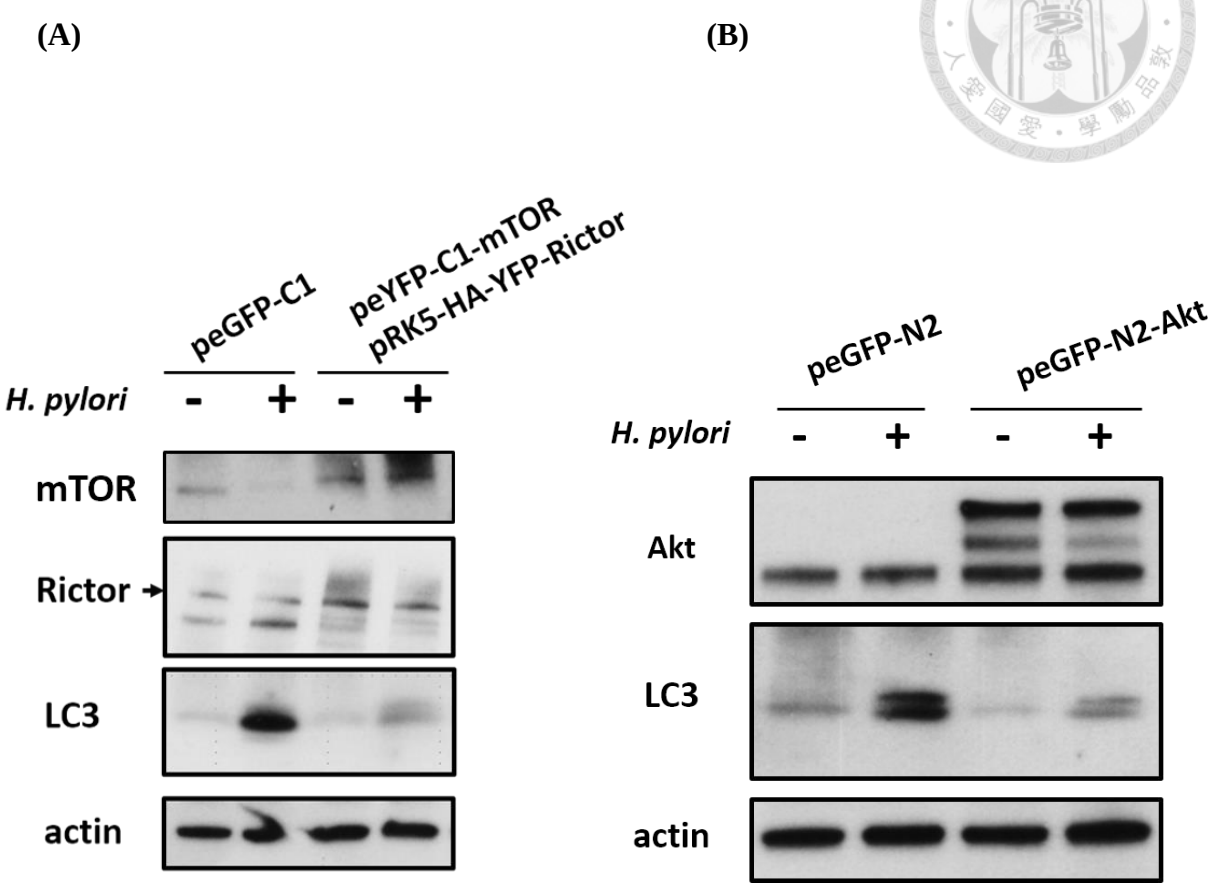

Figure 13. *H. pylori*-induced autophagy was attenuated through activating mTOR-Akt pathway.

(A) Transfected mTOR & Rictor or (B) Akt plasmid in AGS cells for 36 hours and sorted out the GFP^+ /YFP^+ cells to co-culture with *H. pylori* for 12 hours, harvested the cell and detected the signaling changes by Western blot.

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