JBC Papers in Press. Published on May 30, 2017 as Manuscript M117.785170
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M117.785170

# The p90 ribosomal S6 kinase-UBR5 pathway controls toll-like receptor signaling via miRNA-induced translational inhibition of TNF receptor–associated factor 3

Jin Hwa Cho[1,2], Sung Ah Kim[2,3], Yeon-Soo Seo[1], Sung Goo Park[2,3], Byoung Chul Park [2,4†], Jeong-Hoon Kim[3,5†], Sunhong Kim[2,6†]

[1]Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 34141, Republic of Korea
[2]Disease Target Structure Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 34141, Republic of Korea
[3]Department of Functional Genomics, KRIBB school of Bioscience, University of Science and Technology, Daejeon 34113, Republic of Korea
[4] Department of Bio-analytical Science, KRIBB school of Bioscience, University of Science and Technology, Daejeon 34113, Republic of Korea
[5]Personalized Genomic Medicine Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 34141, Republic of Korea
[6]Department of Biomolecular Science, KRIBB school of Bioscience, University of Science and Technology, Daejeon 34113, Republic of Korea

Running Title: p90RSK-UBR5 pathway modulates miRNA action

†Correspondence should be addressed to S. K., Disease Target Structure Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 34141, Republic of Korea; Phone: +82 42 8694278; E-mail: sunhong@kribb.re.kr; J. H. K., Personalized Genomic Medicine Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 34141, Republic of Korea; Phone: +82 42 8694264; E-mail: jhoonkim@kribb.re.kr; B. C. P., Disease Target Structure Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 34141, Republic of Korea; Phone: +82 42 8694260; E-mail: parkbc@kribb.re.kr

Keywords: miRNA, p90RSK, phosphorylation, TRAF3, UBR5, Argonaute, post-translational modification, toll-like receptor



## ABSTRACT

MicroRNAs (miRNAs) are small, noncoding RNAs that post-transcriptionally regulate gene expression. For example, miRNAs repress gene expression by recruiting the miRNA-induced silencing complex (miRISC), a ribonucleoprotein complex that contains miRNA-engaged Argonaute (Ago) and the scaffold protein GW182. Recently, ubiquitin protein ligase E3 component N-recognin 5 (UBR5) has been identified as a component of miRISC. UBR5 directly interacts with GW182 proteins and participates in miRNA silencing by recruiting downstream effectors, such as the translation regulator DEAD-box helicase 6 (DDX6) and transducer of ERBB2.1/2 (Tob1/2), to the Ago-GW182 complex. However, the regulation of miRISC-associated UBR5 remain largely elusive. In the present study, we show that UBR5 down-regulates the levels of TNF receptor–associated factor 3 (TRAF3), a key component of toll-like

receptor signaling, via the miRNA pathway. We further demonstrate that p90 ribosomal S6 kinase (p90RSK) is an upstream regulator of UBR5. p90RSK phosphorylates UBR5 at Thr637, Ser1227, and Ser2483, and the phosphorylation is required for the translational repression of TRAF3 mRNA. Phosphorylated UBR5 co-localized with GW182 and Ago2 in cytoplasmic speckles, which implicated that miRISC is affected by phospho-UBR5. Collectively, these results indicate that the p90RSK-UBR5 pathway stimulates miRNA-mediated translational repression of TRAF3. Our work adds another layer to the regulation of miRISC.

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Micro RNAs (miRNAs) are approximately 21-25 nucleotide (nt) small-noncoding RNAs that post-transcriptionally regulate gene expression. miRNAs are initially transcribed as long primary transcripts that are processed sequentially by Drosha and Dicer enzymes to produce mature miRNA duplex. One strand of the mature miRNAs is incorporated into an Argonaute (Ago) family protein in the miRNA-induced silencing complex (miRISC). After loading, miRNA guides the miRISC to its target mRNA through partial base pairing with complementary sites located mostly in the 3' untranslated region (3'UTR) of target mRNAs and leads to mRNA degradation and/or translational repression (1). The miRISC consists of miRNA, Ago, GW182, and accessory proteins. Association of Ago-GW182 with downstream effector protein determines how to control miRNA-mediated gene suppression. The GW182 proteins play a central role in target mRNA degradation process by interacting with Ago in miRISC (2). GW182 proteins recruit the PAN2-PAN3 and the CAF1-CCR4-NOT deadenylase complexes to miRNA targets through direct interaction with PAN3 and NOT, thereby promoting target mRNA deadenylation and decapping (3). In addition to accelerating mRNA degradation, GW182 is also involved in translational repression through recruiting CCR4-NOT complex and DEAD-box ATPase DDX6 (4,5). GW182 also interact with poly(A)-binding protein (PABP) (6). PABP circularizes mRNA by interacting with both eIF4G (scaffold protein of cap recruit complex, eIF4F) and poly (A) tail, and promotes translational initiation (6,7). The GW182-PABP interaction represses translation by interfering with mRNA circularization (6).

UBR5 (also known as EDD), one of the E6-AP carboxyl terminus (HECT)-type E3 ubiquitin ligase, is originally isolated as a progestin-induced gene in breast cancer cell (8). To date, many proteins have been identified as ubiquitinated substrates and interacting partners for UBR5, through which UBR5 has been shown to be involved in the various cellular processes such as DNA damage responses (9-12), cell cycle progression (13-16), the regulation of transcription (17), translation (18) and interleukin-17 (IL-17) production (19). In $T_H17$ cells, DUBA-UBR5 axis regulates the stability of transcription factor RORγt and IL-17 production (19). DUBA, an ovarian tumor domain containing deubiquitinase, has been identified as a negative regulator of type I interferon (IFN) production (20). DUBA selectively binds and cleaves K63-linked polyubiquitin chains on TRAF3 to suppress Toll-like receptor (TLR)-induced type I interferon (IFN) production but does not affects NF-κB activation (20,21).

In the present study, we show that p90 Ribosomal S6 Kinase (p90RSK)-UBR5 pathway negatively regulates the translation of TRAF3 through miRNA-mediated translational repression. Depletion of UBR5 increased the level of TRAF3 proteins and activity of TRAF3 3'-Untranslated Region (UTR) reporter construct, leading to the activation of TRAF3 signaling. Upon stimulation, activated p90RSK directly phosphorylates UBR5 at Thr637, Ser1227 and Ser2483. In addition, the expression of TRAF3 and KRAS 3'UTR-containing luciferase reporters was also increased by the inhibition of p90RSK, suggesting that p90RSK-UBR5 pathway regulates the action of miRNA in general. Finally, the phosphorylated UBR5 by p90RSK was localized at distinct cytoplasmic speckles with Ago2 and GW182. These results indicate that p90RSK-UBR5 signaling pathway is required for the translational repression by miRNA.

## RESULTS

*UBR5 negatively regulates TRAF3-TBK1 signaling in a DUBA independent manner*-TRAF3 is one of the substrates for DUBA in TLR-mediated type I IFN production (20). To elucidate the roles of UBR5 in the regulation of TRAF3-mediated TLR signaling, we generated HeLa cell lines stably expressing shRNA targeting control or UBR5. Then, the cells was transiently transfected with siRNA targeting control or DUBA to investigate TRAF3 signaling pathway. Depletion of either UBR5 or DUBA in HeLa cells resulted in an increase of TRAF3 protein level (Fig. 1A, the uppermost panel). It has been demonstrated that K63-linked polyubiquitin chains of TRAF3 recruit and activate TANK Binding Kinase 1 (TBK1) upon viral infection (20,22,23). Although overexpression of TRAF3 in epithelial cells can promote the phosphorylation of TBK1 (Supplemental Fig. 1) and the production of IFNβ (24), the increased TRAF3 in DUBA knockdown cells did not induce the phosphorylation of TBK1 (Fig. 1A), consistent with previous study (20). On the contrary, UBR5 knockdown significantly induced the upregulation of TRAF3 protein level and TBK1 activation loop phosphorylation (Fig. 1A). Furthermore, UBR5 depletion increased the activity of IFNβ reporter (Fig. 1B) and the expression of *Ifnb1* mRNA in HeLa cells (Fig. 1C). These data suggest that UBR5 negatively regulates TRAF3 in a DUBA-independent manner.

*UBR5 regulates TRAF3 expression through miRNA-mediated translational repression*- To investigate the mechanism of which UBR5 regulates TRAF3, we examined whether TRAF3 is targeted for ubiquitin-mediated proteasomal degradation by UBR5, because UBR5 belongs to HECT type E3 ubiquitin ligase family (8). To block the ubiquitin-mediated proteasomal degradation pathway and assess TRAF3 level, MG132, a proteasome inhibitor, was used in the stable cell lines expressing control or UBR5 shRNA. The treatment of MG132, however, did not make any significant difference of TRAF3 level in both cell lines (Fig. 2A). Autophagy is another protein degradation system that recognizes the ubiquitinated proteins and degrades them by the lysosomal degradation pathway (25). Chloroquine, a lysosomal degradation inhibitor, also failed to raise the level of TRAF3, but even decreased the amount of TRAF3, while it successfully blocked the degradation of LC3 proteins by autophagy (Fig. 2B). Together, these results suggest that UBR5 activity does not result in the decrease in TRAF3 protein level via proteasome or lysosome.

Alternatively, it has been reported that UBR5 is involved in miRNA-mediated gene silencing pathway (26). *Hong Su* et al. suggested that UBR5 directly interacts with GW182, leading to the miRNA-mediated gene silencing without affecting miRNA biogenesis. To examine whether TRAF3 is regulated through the same pathway, we attempted to inhibit assembly of miRISC by depleting the key component proteins of miRISC such as Argonaute (Ago1, Ago2) and GW182 (TNRC6A) in HeLa cells (Supplemental Fig. 2A-D). Simultaneous knockdown of Ago1 and Ago2 resulted in increased TRAF3 level similar to that of knockdown of UBR5 (Fig. 2C). Moreover, the effect of knockdown of both UBR5 and Ago1/2 were not additive on TRAF3 level (Fig. 2D), suggesting that UBR5 and Ago are on the same signaling pathway to control TRAF3 and the expression of TRAF3 is regulated by miRNA pathway. However, knockdown of TNRC6A failed to increase the amount of TRAF3 proteins. In mammals, GW182 protein family has three paralogs named TNRC6A, B, and C. Because they have redundant functions in miRNA pathway, the depletion of TNRC6A alone may not be enough to inhibit the assembly of miRISC and general miRNA pathway (27). To further investigate whether UBR5 regulates TRAF3 through miRNA-mediated gene silencing pathway, we generated *Firefly* luciferase reporter plasmid (Luc-TRAF3 3' UTR) in which a 5' proximal part of 3' untranslated region (3' UTR) of TRAF3 is attached to 3' end of *Firefly* luciferase gene. This 3' UTR sequence (1313 bp) contains two previously reported target sites of miRNA, miR32 (461-467) (28) and miR422 (1134-1140) (29). The expression of *Firefly*

luciferase is driven by CMV promoter and supposedly suppressed by cellular miRNAs. Depletion of either UBR5 or Ago1/2 in HeLa cells significantly increased the activity of Luc-TRAF3 3'UTR reporter, strongly suggesting that UBR5 is involved in miRNA pathway (Fig. 2E). Additionally, no significant change in the abundance of TRAF3 mRNA was observed in UBR5 knockdown cells, indicating that miRNA-induced mRNA degradation is not involved in the regulation of TRAF3 level by UBR5 (Fig. 2F). To further confirm the miRNA-mediated regulation of TRAF3 by UBR5, the expression of Myc-TRAF3-3'UTR construct was compared with that of Myc-TRAF3 construct containing only coding sequence. In agreement with the luciferase reporter data, the expression of Myc-TRAF3-3'UTR was elevated by UBR5 siRNA, but that of Myc-TRAF3 was not (Fig. 2G). Together, these data suggest that UBR5 regulates TRAF3 expression through miRNA-mediated translational repression.

*RSK phosphorylates UBR5 at Ser2483*-In an attempt to identify upstream regulators of UBR5 involved in miRNA-mediated TRAF3 regulation, we performed the post-translational modifications (PTMs) database search, with PhosphoSite Plus (www.phosphosite.org) (30), to define the PTMs occurred in UBR5. Among 114 potential phosphorylation sites, we noted that Ser2483 of UBR5 belongs to the typical consensus motif (Arg-X-Arg-X-X-Ser/Thr, where X is any amino acid) that is preferentially phosphorylated by AGC kinases, including Akt, p70 Ribosomal S6 Kinase (S6K), Serum and Glucocorticoid-regulated Kinase (SGK), and p90RSK (31), and this motif is evolutionarily conserved through vertebrates (Fig. 3A). To confirm the phosphorylation of UBR5 at Ser2483, we used the anti-pRxRxxS/T antibody, which has been used for the identification of the substrates of AGC kinases. After Flag-UBR5 wild type, Ser2483 to Ala (SA), or Glu (SE) mutant UBR5 was transfected in COS1 cells, phosphorylation status of the immunoprecipitated exogenous UBR5 was examined using the anti-pRxRxxS/T antibody. While SE and SA mutants of UBR5 were not detected by the phospho-antibody, wild type UBR5 was recognized (Fig. 3B). Akt, S6K, SGK, and p90RSK are activated downstream of extracellular growth factor stimuli by PI3K-mTOR or Ras-ERK pathway (31). Indeed, the treatment with EGF, PMA, or FBS induced the phosphorylation of UBR5 in serum-starved HeLa cells (Fig. 3C). It was also demonstrated that the phosphorylation level at Ser2483 of UBR5 reached the peak at 15 min after the EGF stimulation in COS1 and HEK293T cells (Supplemental Fig. 3A and B, respectively). To determine which kinase is responsible for the Ser2483 phosphorylation of UBR5, we used two specific inhibitors, which are GDC0349, an mTOR kinase inhibitor that blocks the activation of Akt, S6K, and SGK, and BI-D1870, a p90RSK inhibitor. As a result, BI-D1870 treatment completely inhibited UBR5 phosphorylation induced by EGF stimulation compared to GDC0349 (Fig. 3D). To confirm the results at endogenous level, the antibody which specifically recognizes Ser2483-phosphorylated UBR5 was raised and used to examine the phosphorylation of endogenous UBR5. The specificity of the antibody was validated by dot blot assay and immunoblot analysis (Supplemental Fig. 3C and D, respectively). Using this anti-phospho-Ser2483 UBR5 antibody (anti-pUBR5 antibody), we observed that the endogenous UBR5 is able to be phosphorylated by EGF, Poly (I:C), and LPS stimulation and that the treatment of BI-D1870 successfully diminished the phosphorylations (Fig. 3E, Supplemental Fig. 3E and F, respectively). Phosphorylation of UBR5 following Poly(I:C) treatment is weaker than the one induced by LPS (Supplemental Fig. 3E and F), suggesting that p90RSK-UBR5 pathway might be affected differentially by viral and bacterial infection.

Then, to examine the possibility that p90RSK directly phosphorylates UBR5, we assessed UBR5 phosphorylation in p90RSK1/2/3 depleted COS1 cells. Simultaneous knockdown of p90RSK1/2/3 by siRNA in COS1 cells significantly attenuated Ser2483 phosphorylation of UBR5 (Fig. 3F). The overexpression of p90RSK in COS1 cells induced the Ser2483 phosphorylation (Fig. 3G). Furthermore, through co-immunoprecipitation analysis, p90RSK was found to interact with UBR5 in HEK293T cells (Fig. 3H). Finally, *in vitro* kinase

assay using Flag-wild type UBR5 or SA mutant UBR5 as a substrate showed that immunoprecipitated Myc-p90RSK is able to phosphorylate wild type UBR5 proteins, but in a lesser extent mutant UBR5 (Fig. 3I). This results suggest that there are another phosphorylation sites of p90RSK in UBR5. Of the sites registered to be phosphorylated, the residue with RxRxxS/T motif is only Ser2483. Instead, there are two more putative p90RSK consensus sites, Thr637 and Ser1227, containing basic residue in -3 and -5 region (K/RxK/RxxS/T) that are preferred motif for p90RSK (31). Thr637 and Ser1227 are located in KxRxxT and KxKxxS motif, respectively and conserved through vertebrates (Supplemental Fig. 3G and H). In *in vitro* kinase assay using UBR5 and p90RSK, Thr637Ala or Ser1227Ala mutation in UBR5 also resulted in the reduction of the p90RSK-mediated phosphorylation of UBR5 (Fig. 3J). Moreover, a UBR5 mutant, which substituted all three sites to Ala (3A), was less phosphorylated by p90RSK than UBR5 SA mutant (Fig. 3K). These data suggested that p90RSK directly phosphorylates UBR5 at Thr637, Ser1227 and Ser2483.

*p90RSK negatively regulates TRAF3-TBK1 signaling by phosphorylating UBR5*-The inhibition of p90RSK activity with BI-D1870 or siRNAs targeting p90RSK1, 2, and 3 in HeLa cells resulted in a substantial increase of TRAF3 level and phosphorylation of TBK1 in the absence of any exogenous stimulation (Fig. 4A and B), indicating that p90RSK activity is required for the repression of TRAF3 level and TBK1 activity as in the case of UBR5. Moreover, BI-D1870 treatment induced IFNβ mRNA (Fig. 4C) and knockdown of p90RSK increased the activity of TRAF3 3'UTR reporter (Fig. 4D). Furthermore, the exogenous expression of constitutively active p90RSK (RSK CA) (32) reduced the expression of TRAF3-3'UTR construct (Fig 4E). Finally, the effects of BI-D1870 treatment and UBR5 knockdown were not additive on TRAF3 level, indicating that p90RSK and UBR5 may be on the same signaling pathway (Fig. 4F and G).

*p90RSK-UBR5 pathway regulates KRAS and p60Katanin expression*-It has been demonstrated that *let-7* miRNA-mediated gene silencing is compromised in UBR5 depleted HeLa cells, which leads to increase the expression of HMGA2, a *let-7* miRNA target gene (26). We confirmed that the activity of *Firefly* luciferase reporter plasmid (Luc-KRAS 3'UTR) in which 3'UTR of KRAS is attached to 3'end of *Firefly* luciferase gene (33) was indeed increased by the knockdown of UBR5 (Fig. 5A) and decreased by the overexpression of UBR5 (Fig. 5G). Next, the inhibition of p90RSK activity by BI-D1870 or depletion of p90RSKs via RNA interference resulted in a significant increase of Luc-KRAS 3'UTR activity (Fig. 5B and C, respectively). In addition, overexpression of RSK CA in HeLa cells significantly decreased the Luc-KRAS 3'UTR activity (Fig. 5D). These data suggested that the regulation of miRNA-mediated gene silencing by p90RSK-UBR5 pathway may be a general phenomenon.

To find other target proteins that are controlled by p90RSK-UBR5 pathway, we tested whether p90RSK can regulate the proteins that are known to be controlled by UBR5. p60Katanin, a microtubule-associated AAA-ATPase, has been known to be one of the substrates for UBR5-DYRK2-DDB1-VPRBP E3 ligase complex (13). In agreement with the previous result, p60Katanin was found to be increased in UBR5 depleted HeLa cells (Supplemental Fig. 4A). Surprisingly, the treatment of BI-D1870 or siRNA transfection of p90RSKs in HeLa cells resulted in increase of p60Katanin protein level (Supplemental Fig. 4B and C). These data suggested the possibility that the abundance of p60Katanin protein may be controlled through p90RSK-UBR5 pathway -regulated miRNA pathway as well as UBR5-DYRK2-DDB1-VPRBP E3 complex.

miRISCs have been mainly localized in cytoplasmic processing bodies (P-bodies) (34) while UBR5 has also been, however, known to be localized in the nucleus (9). To resolve this discrepancy, we examined the localization of UBR5 and p90RSK by cell fractionation. Both of UBR5 and p90RSK were found in the cytoplasm as well as the nucleusand the phosphorylation of UBR5 was elevated by EGF treatment and downregulated

by BI-D1870 treatment (Supplemental Fig. 5A and B). In addition, both of anti-UBR5 and anti-pUBR5 antibodies strongly stained the nucleus, while the prominent cytoplasmic speckles or puncta were also shown by anti-pUBR5 antibody (Supplemental Fig. 5C). To examine the specificity of the antibody, the peptides used for raising the antibody was added to the cells, abrogating the cytoplasmic puncta and the nuclear staining (Supplemental Fig. 5C). It did not seem, however, that Ser2483 phosphorylation of UBR5 induce the nuclear-cytoplasmic shuttling of UBR5 (Supplemental Fig. 5A and B). Furthermore, some of these intriguing speckles colocalized with GW182 and Ago2 which are the components of miRISCs in the cytoplasm and the colocalization signals were decreased following treatment with BI-D1870 (Supplemental Fig. 5D-G). These data suggest that the cytoplasmic p90RSK-UBR5 pathway regulates miRNA-mediated gene silencing.

To further investigate the roles of p90RSK-mediated UBR5 phosphorylation in miRNA pathway, we assess the interaction between GW182 or Ago2 and the phosphorylated UBR5. Phospho-mimic UBR5 mutant with substitution of Glu for the Thr637/Ser1227/Ser2483 (3E) associated more tightly with GW182 and Ago2 than wild type UBR5, but the interaction of UBR5 3SA mutant with GW182 and Ago2 was decreased (Fig. 5E and F). In agreement with these results, UBR5 3E mutant further decreased Luc-KRAS 3'UTR activity than wild type UBR5 in UBR5 depleted HEK293 cells, but UBR5 3A mutant did not (Fig. 5G and Supplemental Fig. 4D). Together these results suggested that phosphorylation of UBR5 at Thr637, Ser1227 and Ser2483 by p90RSK was required for miRNA-mediated gene silencing.

## DISCUSSION

In a recent study, UBR5 was suggested as a substrate of a deubiquitinating enzyme DUBA (also known as OTUD5) (19). DUBA has been first identified as a negative regulator of TRAF3 in TLR signaling (20). Upon the activation of TLR, DUBA is subsequently activated (35) and negatively regulates the induction of type I Interferon by removing the K63-linked ubiquitin chain of TRAF3 (20). DUBA stability is regulated by E3 ubiquitin ligase UBR5 (19) as well as its deubiquitinase activity (35). The question that UBR5 regulates the TLR-mediated TRAF3 signaling via DUBA was raised. Knockdown of UBR5, however, resulted in the increase of TRAF3 proteins, activation of TBK1, and production of IFNβ in spite of the substantial increase of DUBA (Fig. 1). Furthermore, the amount of TRAF3 was controlled by UBR5-regulated miRNA pathway (Fig. 2). These data strongly suggested that DUBA and UBR5 converge on TRAF3 signaling in parallel independently of DUBA-UBR5 axis presented in the previous study (19).

TLRs activates mitogen-activated protein kinase (MAPK) pathway, including ERK, JNK, and p38, to induce the genes encoding inflammatory cytokines and generate immune responses (36). In macrophages and myeloid dendritic cells, it was reported that TPL2-mediated ERK activation negatively regulates IFNβ production (37). It is very well known that p90RSK is directly phosphorylated and activated by ERK (38), however, the functions and substrates of p90RSK in immune response remain elusive (36). In present study, we found that the p90RSK directly phosphorylates UBR5 at Thr637, Ser1227 and Ser2483 in response to the activation of TLRs as well as EGF receptor (Fig. 3 and Supplemental Fig. 3) and the inhibition of UBR5 phosphorylation abolished the translational repression of TRAF3 and KRAS repression (Fig. 4 and 5). In terms of TLR signaling, these results propose the possibility that pathogen-associated molecular patterns (PAMPs) may activate TRAF3-TBK1 pathway via K63-ubiquitination, as well as fine-tune the strength of the signaling via p90RSK-UBR5 pathway-mediated suppression of TRAF3 expression and DUBA-mediated deubiquitination of TRAF3. miRNA pathway can be regulated through controlling the activity of miRISC as well as biogenesis of miRNAs (39). To date, a number of upstream regulators and PTMs of Ago protein have been identified. PTMs of Ago protein, including phosphorylation, hydroxylation, ubiquitination, altered the stability, ability of binding to small RNA molecules, and cellular

localization of Ago (40-43). In present study, we suggested another regulator mechanism for regulating miRISC activity by phosphorylating UBR5 (Fig. 6).

UBR5 3A mutants are still able to be phosphorylated by activated p90RSK in *in vitro* kinase assay (Fig. 3K). This result raise a possibility that there are other phosphorylation sites in UBR5. There are two evolutionarily conserved RxRxxS/T motif in human UBR5, Ser26 and Ser2483. Phospho-RxRxxS/T antibody, however, did not recognize the Ser2483 mutants of UBR5 (Fig. 3B), indicating that Ser26 is barely or not phosphorylated by p90RSK. Although there may be more phosphorylation sites of UBR5 by p90RSK, it has been shown that 3E mutant interacts with GW182 and Ago2 stronger than wild type and 3A mutant is weaker than wild type and 3E mutant in terms of the association with miRISC and translational repression (Fig. 5E-G). These data collectively suggest that three residues are the main regulatory sites in UBR5.

UBR5 proteins have been shown to reside in the nucleus (9). In contrast, miRISCs are mainly localized in cytoplasmic processing bodies (P-bodies) (34). The nuclear cytoplasmic fractionation and immunocytochemistry showed that a part of phosphorylated UBR5 is colocalized with a subset of miRISC proteins at the cytoplasmic punctate structure (Supplemental Fig. 5). These findings suggest the possibility that there are UBR5-specific miRISC sets. Further study will be needed to elucidate the components of p90RSK-UBR5-containing miRISCs and their target miRNA-mRNA sets.

Together our findings have established p90RSK-UBR5 pathway as a positive regulator of miRNA-mediated gene silencing pathway and a negative regulatory mechanism of TLR signaling.

## EXPERIMENTAL PROCEDURES

*Antibodies and reagents*-Anti-UBR5 antibody (8755), TRAF3 antibody (4729), TBK1 antibody (3504), phospho-TBK1 Ser172 antibody (5483), phospho-Akt substrate (RXRXX(p)S/(p)T) antibody (10001), RSK1 antibody (8408) and RSK2 antibody (5528) were obtained from Cell Signaling Technology. Anti-DUBA antibody (ab176727), GW182 antibody (ab70522) and Ago2 antibody (ab57113) were obtained from Abcam. Anti-RSK3 antibody (sc1431), DDX6 antibody (sc376433) and Myc antibody (sc40) were obtained from Santa Cruz Biotechnology. Anti-LC3 antibody (PM036) was obtained from MBL. Anti-HA antibody (11867423001) was obtained from Roche. Anti-Flag antibody (F1804) and Tubulin antibody (T5168) were obtained from Sigma Aldrich. Polyclonal anti-phospho-UBR5 Ser2483 antibody was developed in rabbits using a synthetic phospho-peptide derived from 2476-2486 amino acid of human UBR5 as an immunogen and the serum was purified by affinity chromatography by AbClon (Rep. of Korea). Anti-Flag M2 affinity gel (A2220) and anti-c-Myc agarose gel (A7470) were obtained from Sigma Aldrich. Protein G Sepharose (17-0618-01) was obtained from GE Healthcare. MG132 (C2211), Chloroquine (C6628), EGF (E9644), Phorbol 12-myristate 13-acetate (PMA, P1585) and Polyinosinic-polycytidylic acid potassium salt (poly(I:C), I3036) were obtained from Sigma Aldrich. BI-D1870 (S2843) and GDC0349 (S8040) were obtained from Selleckchem.

*Cell culture*-HeLa, COS1 and HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin 100units/ml, streptomycin 100μg/ml, amphotericin B 0.25μg/ml (Gibco) in a humidified atmosphere of 5% $CO_2$ in air at 37°C.

*Plasmids and transfection*-Myc-GFP-TNRC6A (41999) and GFP-hAgo2 (11590) were obtained from Addgene. Cells were transfected with various plasmids using X-treamGENE HP DNA Transfection Reagent (Roche) according to the manufacturer's instruction.

*RNA interference*-siRNA against Ago1 (siRNA no.1046447), Ago2 (siRNA no.1046457), TNRC6A (siRNA no.1154883) and negative control siRNA (SN-1003) were obtained from Bioneer (Rep. of Korea). siRNA against DUBA (L-013823-00) was obtained from Dharmacon. siRNA against UBR5 was obtained from Santa Cruz

Biotechnology (sc-43744) and Bioneer (siRNA no.1045520). siRNA against RSK1, 2 and 3 were obtained from Dharmacon (L-003025-00, L-003026 and L-004663-00) and Bioneer (siRNA no.1131587, 1131617 and 1131598). For RNA interference, cells were transfected with various siRNAs using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instruction. After 48h to 72h incubation, cells were harvested and analyzed.

*Lentivirus production and transduction*-For producing lentiviral particles, HEK293T cells were transfected with a lentiviral plasmid pLKO.1 containing UBR5 3'UTR targeting shRNA (TRCN0000226459, Sigma Aldrich) or non-targeting shRNA (SHC202, Sigma Aldrich), psPAX2, and pMD2.G at a ratio of 1 : 0.75 : 0.25. After 72h transfection, culture supernatant containing lentivirus were collected and filtered through a 0.45μm sterile filter to remove cell debris. For generating stably expressed UBR5 or negative control shRNA HeLa cells, cells were treated with the viral supernatant and hexadimethrine bromide 8μg/ml (H9268, Sigma Aldrich) and incubate for 48hr. After viral transduction, cells were treated and selected with 2μg/ml puromycin.

*Immunoprecipitation and immunoblotting* -Cells were lysed using lysis buffer (50mM HEPES KOH pH7.4, 40mM NaCl, 1mM EDTA, 1mM EGTA, 10mM Na pyrophosphate, 10mM Na β-glycerophosphate, 50mM NaF, 1mM NaVO4, 1% Triton X-100) containing protease inhibitor (05056489001, Roche). The lysate was clarified by centrifuging at 13,000 rpm, 4°C for 15 min. The supernatant was quantified and incubated with antibody and beads at 4°C for 2h or overnight. After incubation, beads were washed twice in lysis buffer, twice in wash buffer (50mM HEPES KOH pH7.4, 500mM NaCl, 1mM EDTA, 1mM EGTA, 10mM Na pyrophosphate, 10mM Na β-glycerophosphate, 50mM NaF, 1mM $NaVO_4$, 1% Triton X-100, protease inhibitor) and once in lysis buffer. In coIP experiments, beads were washed three times in lysis buffer. Elution was performed using 2X Laemmli buffer, and samples were separated using SDS-PAGE. After transfer to NC membrane, immunoblotting was performed using the indicated antibodies (44).

*Luciferase assay*-HeLa cells were transfected with indicated siRNAs. After 24h transfection, cells were transfected with indicated luciferase reporter plasmids and pRL-CMV for 48h. Luciferase activities were measured with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instruction.

*Real-time PCR analysis*-Total RNA was extracted from siRNAs- or chemicals-treated cells using RNeasy Plus Mini Kit (QIAGEN) and 2μg of total RNA was reverse transcribed using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instruction. Real-time PCR was performed using Solg 2X Real-Time PCR Smart mix (SRH71-M40H, SolGent) and gene-specific primers. *GAPDH* was used for normalization. The sequence of primers used for real-time PCR is shown in Supplemental Table 2.

*In vitro kinase assay*-HEK293 cells were transfected respectively with plasmids encoding Flag-tagged-wild type UBR5, -mutant UBR5, or Myc-tagged avian RSK. After 48h transfection, Flag-UBR5 overexpressed cells were serum starved for 24h and then treated with BI-D1870 10μM for 5h. Myc-RSK overexpressed cells were serum starved for 24h and then stimulated with EGF 100ng/ml (or not, as indicated) for 30min. Cells were rinsed with ice-cold PBS and lysed using lysis buffer. The clarified lysates were immunoprecipitated with anti-Flag-agarose gel (for Flag-UBR5) or anti-c-Myc-agarose gel (for Myc-RSK) at 4°C for 3h. The beads were washed twice in lysis buffer, twice in wash buffer, once in lysis buffer and once in kinase buffer (25mM HEPES-KOH pH7.4, 10mM $MgCl_2$, 3mM β-mercaptoethanol, 0.1mg/ml BSA, 1mM DTT). In the last washing step, Flag-UBR5 (or negative control) binding beads and Myc-RSK (either stimulated or not with EGF) binding beads were combined as indicated. The kinase assay was performed by adding kinase reaction buffer (25mM HEPES-KOH pH7.4, 10mM $MgCl_2$, 3mM β-mercaptoethanol, 0.1mg/ml BSA, 1mM DTT, 50μM ATP, 10μCi [$\gamma$-$^{32}$P] ATP) and incubating at 30°C for 30min. Reactions were stopped by adding

Laemmli buffer and boiling at 100°C for 5min. Reactions were separated using SDS-PAGE and the gel was stained with Coomassie blue solution. After drying the stained gel on Whatman 3MM paper, $^{32}P$ incorporation was determined by film exposure.

*Immunofluorescence microscopy*-HeLa cells (or plasmids-transfected HeLa cells) were seeded in μ-Slide 8 well (80826, Ibidi) at a densities of 5 X $10^4$ cells/well. The next day, cells were washed with warm PBS and then fixed with 4% paraformaldehyde in PBS for 20 min at RT. After wash out with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min at RT. And then cells were washed with PBS and blocked with Image-iT FX Signal Enhancer (136933, Molecular Probes) for 30 min at RT. After blocking, cells were washed with PBS and stained with the indicated primary antibodies at a ratio of 1:100 in 4% BSA/PBS for 2h at RT followed by secondary antibodies (Goat anti-Rabbit IgG Alexa Fluor 488 (11008, Invitrogen), Goat anti-Rabbit IgG Alexa Fluor 546 (A11010, Invitrogen), Goat anti-Mouse IgG Alexa Fluor 488 (A11001, Invitrogen)) at a ratio of 1:1000 in 4% BSA/PBS for 1h at RT. After staining, cells were washed with PBS and mounted in Fluoroshield Mounting Medium with DAPI (ab104139, Abcam). Fluorescence was detected using a laser scanning microscope LSM 880 (ZEISS).

*Proximity ligation assay*-HeLa cells were seeded in μ-Slide 8 well at a densities of 5 X $10^4$ cells/well. Cells were fixed, permeabilized, and stained with primary antibodies according to the method described in Immunofluorescence microscopy. Proximity ligation assay was performed using Duolink In Situ Red Starter Kit Mouse/Rabbit (DUO92101, Sigma Aldrich) according to the manufacturer's instruction. The Fluorescence signals were measured using a ZEISS LSM 880.

*Statistical analysis*-All data were obtained from at least three independent experiments and expressed as means ± SD. Statistical analysis were performed using Microsoft Excel Student's *t*-test (two-tailed), with *P*-value <0.05 considered significant.

**Acknowledgements**
This work was supported by the grant (CAP-15-11-KRICT) from National Research Council of Science and Technology, Ministry of Science, ICT, and future planning and the grant from KRIBB Research Initiative Program.

**Conflict of interest**
The authors declare that they have no conflict of interest.

**Author contributions**
JHC conducted most of the experiments regarding p90RSK-UBR5 pathway in miRNA pathway and SAK mostly worked on the cloning and luciferase assays. YS and SGP supported design of some of the experiments. BCP, JHK, and SK contributed to experimental design and supervision, and along with other authors wrote the manuscript.

## References


1. Carthew, R. W., and Sontheimer, E. J. (2009) Origins and Mechanisms of miRNAs and siRNAs. *Cell* **136**, 642-655
2. Eulalio, A., Huntzinger, E., and Izaurralde, E. (2008) GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. *Nat Struct Mol Biol* **15**, 346-353
3. Braun, J. E., Huntzinger, E., Fauser, M., and Izaurralde, E. (2011) GW182 proteins directly recruit cytoplasmic deadenylase complexes to miRNA targets. *Mol Cell* **44**, 120-133
4. Chu, C. Y., and Rana, T. M. (2006) Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. *PLoS Biol* **4**, e210
5. Rouya, C., Siddiqui, N., Morita, M., Duchaine, T. F., Fabian, M. R., and Sonenberg, N. (2014) Human DDX6 effects miRNA-mediated gene silencing via direct binding to CNOT1. *RNA* **20**, 1398-1409
6. Zekri, L., Huntzinger, E., Heimstadt, S., and Izaurralde, E. (2009) The silencing domain of GW182 interacts with PABPC1 to promote translational repression and degradation of microRNA targets and is required for target release. *Mol Cell Biol* **29**, 6220-6231
7. Sonenberg, N., and Hinnebusch, A. G. (2009) Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell* **136**, 731-745
8. Callaghan, M. J., Russell, A. J., Woollatt, E., Sutherland, G. R., Sutherland, R. L., and Watts, C. K. (1998) Identification of a human HECT family protein with homology to the Drosophila tumor suppressor gene hyperplastic discs. *Oncogene* **17**, 3479-3491
9. Henderson, M. J., Russell, A. J., Hird, S., Munoz, M., Clancy, J. L., Lehrbach, G. M., Calanni, S. T., Jans, D. A., Sutherland, R. L., and Watts, C. K. (2002) EDD, the human hyperplastic discs protein, has a role in progesterone receptor coactivation and potential involvement in DNA damage response. *J Biol Chem* **277**, 26468-26478
10. Henderson, M. J., Munoz, M. A., Saunders, D. N., Clancy, J. L., Russell, A. J., Williams, B., Pappin, D., Khanna, K. K., Jackson, S. P., Sutherland, R. L., and Watts, C. K. (2006) EDD mediates DNA damage-induced activation of CHK2. *J Biol Chem* **281**, 39990-40000
11. Honda, Y., Tojo, M., Matsuzaki, K., Anan, T., Matsumoto, M., Ando, M., Saya, H., and Nakao, M. (2002) Cooperation of HECT-domain ubiquitin ligase hHYD and DNA topoisomerase II-binding protein for DNA damage response. *J Biol Chem* **277**, 3599-3605
12. Gudjonsson, T., Altmeyer, M., Savic, V., Toledo, L., Dinant, C., Grofte, M., Bartkova, J., Poulsen, M., Oka, Y., Bekker-Jensen, S., Mailand, N., Neumann, B., Heriche, J. K., Shearer, R., Saunders, D., Bartek, J., Lukas, J., and Lukas, C. (2012) TRIP12 and UBR5 suppress spreading of chromatin ubiquitylation at damaged chromosomes. *Cell* **150**, 697-709
13. Maddika, S., and Chen, J. (2009) Protein kinase DYRK2 is a scaffold that facilitates assembly of

an E3 ligase. *Nat Cell Biol* **11**, 409-419
14. Jung, H. Y., Wang, X., Jun, S., and Park, J. I. (2013) Dyrk2-associated EDD-DDB1-VprBP E3 ligase inhibits telomerase by TERT degradation. *J Biol Chem* **288**, 7252-7262
15. Benavides, M., Chow-Tsang, L. F., Zhang, J., and Zhong, H. (2013) The novel interaction between microspherule protein Msp58 and ubiquitin E3 ligase EDD regulates cell cycle progression. *Biochim Biophys Acta* **1833**, 21-32
16. Smits, V. A. (2012) EDD induces cell cycle arrest by increasing p53 levels. *Cell Cycle* **11**, 715-720
17. Chen, H. W., Yang, C. C., Hsieh, C. L., Liu, H., Lee, S. C., and Tan, B. C. (2013) A functional genomic approach reveals the transcriptional role of EDD in the expression and function of angiogenesis regulator ACVRL1. *Biochim Biophys Acta* **1829**, 1309-1319
18. Yoshida, M., Yoshida, K., Kozlov, G., Lim, N. S., De Crescenzo, G., Pang, Z., Berlanga, J. J., Kahvejian, A., Gehring, K., Wing, S. S., and Sonenberg, N. (2006) Poly(A) binding protein (PABP) homeostasis is mediated by the stability of its inhibitor, Paip2. *EMBO J* **25**, 1934-1944
19. Rutz, S., Kayagaki, N., Phung, Q. T., Eidenschenk, C., Noubade, R., Wang, X., Lesch, J., Lu, R., Newton, K., Huang, O. W., Cochran, A. G., Vasser, M., Fauber, B. P., DeVoss, J., Webster, J., Diehl, L., Modrusan, Z., Kirkpatrick, D. S., Lill, J. R., Ouyang, W., and Dixit, V. M. (2015) Deubiquitinase DUBA is a post-translational brake on interleukin-17 production in T cells. *Nature* **518**, 417-421
20. Kayagaki, N., Phung, Q., Chan, S., Chaudhari, R., Quan, C., O'Rourke, K. M., Eby, M., Pietras, E., Cheng, G., Bazan, J. F., Zhang, Z., Arnott, D., and Dixit, V. M. (2007) DUBA: a deubiquitinase that regulates type I interferon production. *Science* **318**, 1628-1632
21. Kondo, T., Kawai, T., and Akira, S. (2012) Dissecting negative regulation of Toll-like receptor signaling. *Trends Immunol* **33**, 449-458
22. Guo, B., and Cheng, G. (2007) Modulation of the interferon antiviral response by the TBK1/IKKi adaptor protein TANK. *J Biol Chem* **282**, 11817-11826
23. Tseng, P. H., Matsuzawa, A., Zhang, W., Mino, T., Vignali, D. A., and Karin, M. (2010) Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines. *Nat Immunol* **11**, 70-75
24. Perkins, D. J., Polumuri, S. K., Pennini, M. E., Lai, W., Xie, P., and Vogel, S. N. (2013) Reprogramming of murine macrophages through TLR2 confers viral resistance via TRAF3-mediated, enhanced interferon production. *PLoS Pathog* **9**, e1003479
25. Mizushima, N. (2007) Autophagy: process and function. *Genes Dev* **21**, 2861-2873
26. Su, H., Meng, S., Lu, Y., Trombly, M. I., Chen, J., Lin, C., Turk, A., and Wang, X. (2011) Mammalian hyperplastic discs homolog EDD regulates miRNA-mediated gene silencing. *Mol Cell* **43**, 97-109

27. Eulalio, A., Tritschler, F., and Izaurralde, E. (2009) The GW182 protein family in animal cells: new insights into domains required for miRNA-mediated gene silencing. *RNA* **15**, 1433-1442
28. Mishra, R., Chhatbar, C., and Singh, S. K. (2012) HIV-1 Tat C-mediated regulation of tumor necrosis factor receptor-associated factor-3 by microRNA 32 in human microglia. *J Neuroinflammation* **9**, 131
29. Gu, H., Yu, J., Dong, D., Zhou, Q., Wang, J. Y., and Yang, P. (2015) The miR-322-TRAF3 circuit mediates the pro-apoptotic effect of high glucose on neural stem cells. *Toxicol Sci* **144**, 186-196
30. Hornbeck, P. V., Zhang, B., Murray, B., Kornhauser, J. M., Latham, V., and Skrzypek, E. (2015) PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Res* **43**, D512-520
31. Pearce, L. R., Komander, D., and Alessi, D. R. (2010) The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol* **11**, 9-22
32. Frodin, M., Jensen, C. J., Merienne, K., and Gammeltoft, S. (2000) A phosphoserine-regulated docking site in the protein kinase RSK2 that recruits and activates PDK1. *EMBO J* **19**, 2924-2934
33. Johnson, S. M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K. L., Brown, D., and Slack, F. J. (2005) RAS is regulated by the let-7 microRNA family. *Cell* **120**, 635-647
34. Ding, L., and Han, M. (2007) GW182 family proteins are crucial for microRNA-mediated gene silencing. *Trends Cell Biol* **17**, 411-416
35. Huang, O. W., Ma, X., Yin, J., Flinders, J., Maurer, T., Kayagaki, N., Phung, Q., Bosanac, I., Arnott, D., Dixit, V. M., Hymowitz, S. G., Starovasnik, M. A., and Cochran, A. G. (2012) Phosphorylation-dependent activity of the deubiquitinase DUBA. *Nat Struct Mol Biol* **19**, 171-175
36. Arthur, J. S., and Ley, S. C. (2013) Mitogen-activated protein kinases in innate immunity. *Nat Rev Immunol* **13**, 679-692
37. Kaiser, F., Cook, D., Papoutsopoulou, S., Rajsbaum, R., Wu, X., Yang, H. T., Grant, S., Ricciardi-Castagnoli, P., Tsichlis, P. N., Ley, S. C., and O'Garra, A. (2009) TPL-2 negatively regulates interferon-beta production in macrophages and myeloid dendritic cells. *J Exp Med* **206**, 1863-1871
38. Anjum, R., and Blenis, J. (2008) The RSK family of kinases: emerging roles in cellular signalling. *Nat Rev Mol Cell Biol* **9**, 747-758
39. Leung, A. K., and Sharp, P. A. (2010) MicroRNA functions in stress responses. *Mol Cell* **40**, 205-215
40. Qi, H. H., Ongusaha, P. P., Myllyharju, J., Cheng, D., Pakkanen, O., Shi, Y., Lee, S. W., Peng, J., and Shi, Y. (2008) Prolyl 4-hydroxylation regulates Argonaute 2 stability. *Nature* **455**, 421-424

41. Zeng, Y., Sankala, H., Zhang, X., and Graves, P. R. (2008) Phosphorylation of Argonaute 2 at serine-387 facilitates its localization to processing bodies. *Biochem J* **413**, 429-436
42. Rudel, S., Wang, Y., Lenobel, R., Korner, R., Hsiao, H. H., Urlaub, H., Patel, D., and Meister, G. (2011) Phosphorylation of human Argonaute proteins affects small RNA binding. *Nucleic Acids Res* **39**, 2330-2343
43. Rybak, A., Fuchs, H., Hadian, K., Smirnova, L., Wulczyn, E. A., Michel, G., Nitsch, R., Krappmann, D., and Wulczyn, F. G. (2009) The let-7 target gene mouse lin-41 is a stem cell specific E3 ubiquitin ligase for the miRNA pathway protein Ago2. *Nat Cell Biol* **11**, 1411-1420
44. Kim, S., Johnson, W., Chen, C., Sewell, A. K., Bystrom, A. S., and Han, M. (2010) Allele-specific suppressors of lin-1(R175Opal) identify functions of MOC-3 and DPH-3 in tRNA modification complexes in Caenorhabditis elegans. *Genetics* **185**, 1235-1247

**Footnotes**

The abbreviations used are: miRNAs, MicroRNAs; miRISC, miRNA-Induced Silencing Complex; Ago, Argonaute; p90RSK, p90 Ribosomal S6 Kinase; nt, nucleotide; 3'UTR, 3'untranslated region; PABP, poly(A)-binding protein; HECT, E6-AP carboxyl terminus; IL-17, interleukin-17; IFN, interferon; TLR, Toll-like receptor; TBK1, TANK Binding Kinase 1; PTMs, post-translational modifications;S6K, p70 Ribosomal S6 Kinase; SGK, Serum and Glucocorticoid-regulated Kinase; P-body, processing bodies; IP, immunoprecipitation; CBS, coomassie blue staining; Luc, luciferase; qPCR, real-time PCR; WCL, whole cell lysates.

## FIGURE LEGENDS

**FIGURE 1.** UBR5 negatively regulates TRAF3-TBK1 signaling by DUBA-independent mechanism.

A. Immunoblot analysis using whole-cell lysate of control or UBR5 shRNA stable HeLa cell lines transfected with control or DUBA siRNA for 48h. pTBK1 indicates phospho-S172 TBK1 antibody. All the immunoblots were completed using the same cell lysates. Data are representatives of three independent experiments.

B. The dual luciferase assay for IFNβ gene promoter were completed from the HeLa cell lysates 72h after the transfection with control or UBR5 siRNA as described in Experimental Procedures. Data are shown as the mean ± SD of four samples from a representative experiments performed three times.

C. qPCR analysis of *Ifnb1* mRNA level in control or UBR5 siRNA transfected HeLa cells. Data are shown as the mean ± SD of four samples from a representative experiments performed three times.

**FIGURE 2.** UBR5 regulates TRAF3 expression through miRNA-mediated translational repression.

A, B. Immunoblot analysis of indicated proteins using the whole-cell lysates of HeLa cell lines stably expressing control or UBR5 shRNA treated with DMSO or MG132 (10μM) for 6h (A), or Chloroquine (100nM) for indicated time periods (B).

C, D. Immunoblot analysis of indicated proteins using the whole-cell lysates of HeLa cells transfected with siRNAs against Ago1/2, GW182, UBR5, or control siRNA for 72h.

E. Luciferase assay of TRAF3 3'UTR luciferase activity using lysate of HeLa cells that were transfected with siRNAs against UBR5, Ago1, 2, or control siRNA. Data are shown as the mean ± SD of duplicate samples from a representative experiments performed three times.

F. qPCR analysis of *UBR5* and *TRAF3* mRNA level in control and UBR5 siRNA transfected HeLa cells. Data are shown as the mean ± SD of duplicate samples from a representative experiments performed three times. ($P$ = n.s., Student's $t$-test)

G. Immunoblot analysis of exogenous Myc-tagged TRAF3 containing 3'UTR region (Myc-TRAF3U) or not (Myc-TRAF3) in HeLa cells that were transfected with control or UBR5 siRNA for 72h. Data are representative of at least two independent experiments.

**FIGURE 3.** p90RSK phosphorylates UBR5 at multiple sites.

A. Conservation of RxRxxS motif of UBR5 Ser2483 among various species.

B. Flag-wild-type UBR5 (WT) or the indicated UBR5 mutant (S2483A: SA, S2483E: SE) were transfected in COS1 cells. The Flag-UBR5 were isolated by immunoprecipitation and subjected to immunoblot analysis for phosphorylation using indicated antibodies (upper and middle panels). The cell lysates were subjected to direct immunoblot (bottom panel, Input: 2% of the total lysates).

C. After 24h serum-starvation, the HeLa cells transfected with Flag-UBR5 were stimulated with EGF (100ng/ml), PMA (50ng/ml), or fetal bovine serum (FBS, 20%) for 15min. The lysates were subjected to immunoprecipitation, followed by immunoblot analysis using the indicated antibodies.

D. After 24h serum-starvation, the COS1 cells were pretreated with GDC0349 (1μM) or BI-D1870 (10μM) for 30min prior to EGF (100ng/ml) stimulation for 15min. Flag-UBR5 were isolated by immunoprecipitation and subjected to immunoblot analysis using the indicated antibodies.

E. The serum-starved COS1 cells were stimulated with EGF for 15min in the absence or presence of BI-D1870 (for 30min). The cell lysates were subjected to the immunoblots using indicated antibodies.

F. COS1 cells were transfected with control siRNA or p90RSK1, 2, 3 siRNA and empty vector or Flag-UBR5 as indicated. The Flag-UBR5 were isolated by immunoprecipitation and subjected to immunoblot analysis using indicated antibodies (IP) and the cell lysates were subjected to direct immunoblot with the indicated antibodies (Input).

G. COS1 cells were cotransfected with empty vector or Myc-avian p90RSK and empty vector or Flag-UBR5 as indicated. The Flag-UBR5 were isolated by immunoprecipitation and subjected to immunoblot analysis using indicated antibodies (IP) and the cell lysates were subjected to direct immunoblot with the indicated antibodies (Input).

H. COS1 cells were cotransfected with empty vector or HA-human p90RSK1 (hRSK1) and empty vector or Flag-UBR5 as indicated. The Flag-UBR5 were isolated by immunoprecipitation and subjected to immunoblot analysis with the indicated antibodies to detect UBR5-p90RSK1 interaction (IP) and the cell lysates were subjected to direct immunoblot with same antibodies (Input).

I-K. *In vitro* kinase assay using Flag-UBR5 and Myc-avian p90RSK (I), Flag-wild type (WT), -Thr637Ala (T637A), -Ser1227Ala (S1227A), -Ser2483Ala (S2483A) UBR5 and Myc-avian p90RSK (J), Flag-WT, S2483A (1A), T637/S1227/S2483A (3A) and Myc-avian p90RSK (K) as described in Experimental Procedures. CBS stands for Coomassie Blue Staining.

**FIGURE 4.** p90RSK negatively regulates TRAF3-TBK1 signaling via UBR5.

A, B. Immunoblot analysis of indicated proteins using the whole cell lysates of HeLa cells that were treated with DMSO or BI-D1870 for 12h (A), or transfected with siRNAs against p90RSK1, 2, 3 and control siRNA for 48h (B). All the immunoblots were completed using the same cell lysates.

C. qPCR analysis of *Ifnb1* mRNA level using HeLa cells that were treated with DMSO or BI-D1870 for 12h. Data are shown as the mean ± SD of duplicate samples from a representative experiments performed three times.

D. Dual luciferase assays were performed using TRAF3 3'UTR reporter in HeLa cells transfected with siRNAs against p90RSK-1, -2, -3, Ago-1, -2 and control siRNA. Data are shown as the mean ± SD of duplicate samples from a representative experiments performed three times.

E. HeLa cells were cotransfected with empty vector or Myc-TRAF3U and empty vector or HA-constitutively active human p90RSK1 (RSK CA) as indicated for 48h. After 12h serum-starvation, the cells lysates were subjected to immunoblot with indicated antibodies.

F. Immunoblot analysis of indicated proteins using DMSO or BI-D1870 (for 12h) treated HeLa cells that were transfected with siRNAs against control or UBR5 for 48h.

G. Densitometry analysis of immunoblot bands of (F) from four independent experiments. TRAF3 bands were normalized to tubulin bands. Data are shown as the mean ± SD from four independent experiments (**P*=0.005, ***P*=0.0005, ****P*=0.002, and *P*=n.s., Student's *t*-test).

**FIGURE 5.** p90RSK-UBR5 pathway regulates KRAS and p60Katanin expression.

A. Dual luciferase assays using KRAS 3'UTR reporter were carried out using the whole cell lysates of control or UBR5 shRNA stable HeLa cells. Data are shown as the mean ± SD of duplicate samples from a representative experiments performed three times.

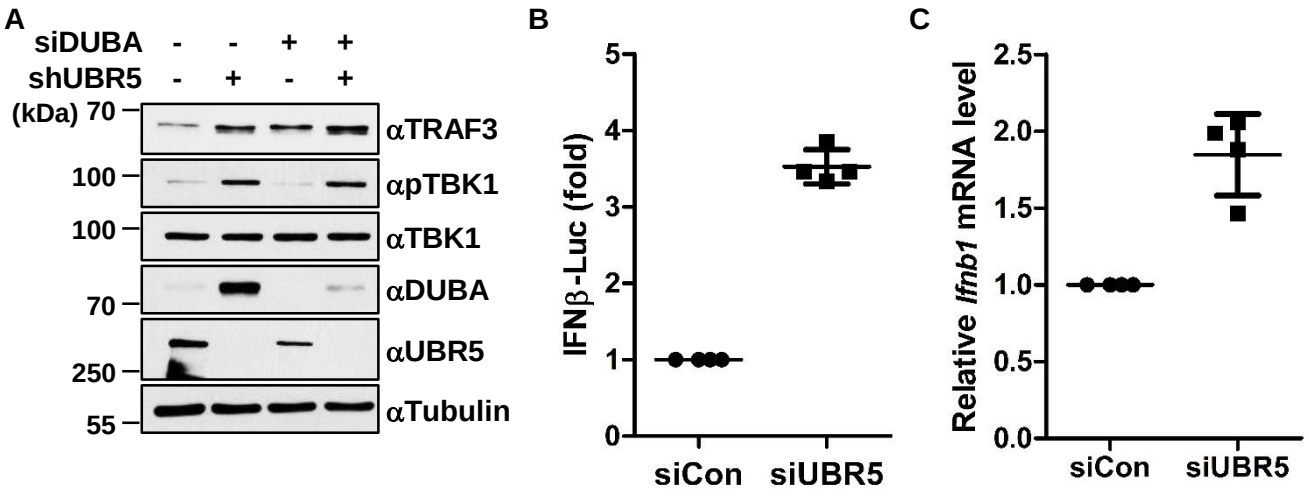
B-D. KRAS 3'UTR reporter assays were performed using the cells treated with DMSO or BI-D1870 for 12h (B), transfected with control or p90RSK1,2,3 siRNAs for 48h (C), or transfected with empty vector or HA-RSK CA for 48h (D). Data are shown as the mean ± SD of duplicate samples from a representative experiments performed two times.

E. HEK293 cells were cotransfected with empty vector (-) or Flag-wild type (+),3E, 3A mutant UBR5 and Myc-GFP-GW182 as indicated. After 48h, the Myc-GFP-GW182 were immunoprecipitated using anti-Myc antibody and subjected to immunoblot analysis with the indicated antibodies to detect UBR5 and GW182 interaction (IP) and the cell lysates were subjected to direct immunoblot with same antibodies (Input).

F. HEK293 cells were cotransfected with empty vector (-) or Flag-wild type (+),3E, 3A mutant UBR5 and HA-Ago2 as indicated. After 48h, the Flag-UBR5 were immunoprecipitated using anti-Flag antibody and subjected to immunoblot analysis with the indicated antibodies to detect UBR5 and Ago2 interaction (IP) and the cell lysates were subjected to direct immunoblot with same antibodies (Input).

G. Luciferase assay of KRAS 3'UTR luciferase activity using control or UBR5 shRNA stable HEK293 cell lysates that were cotransfected with Luc-KRAS 3'UTR, pRL-CMV and empty vector (-), Flag-wild type UBR5 (WT), 3E, 3A mutant. Data are shown as the mean ± SD of duplicate samples from a representative experiments performed two times. (**P*=0.03, ***P*=0.0006, ****P*=0.005, Student's *t*-test).

**FIGURE 6.** Schematic overview for p90RSK-mediated regulation of UBR5 containing miRISC.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shUBR5 - + - +
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αTRAF3
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αpTBK1
100
αTBK1
αDUBA
70
αUBR5
250
αTubulin
55
B
IFNβ-Luc (fold)
0
1
2
3
4
5
siCon
siUBR5
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Relative Ifnb1 mRNA level
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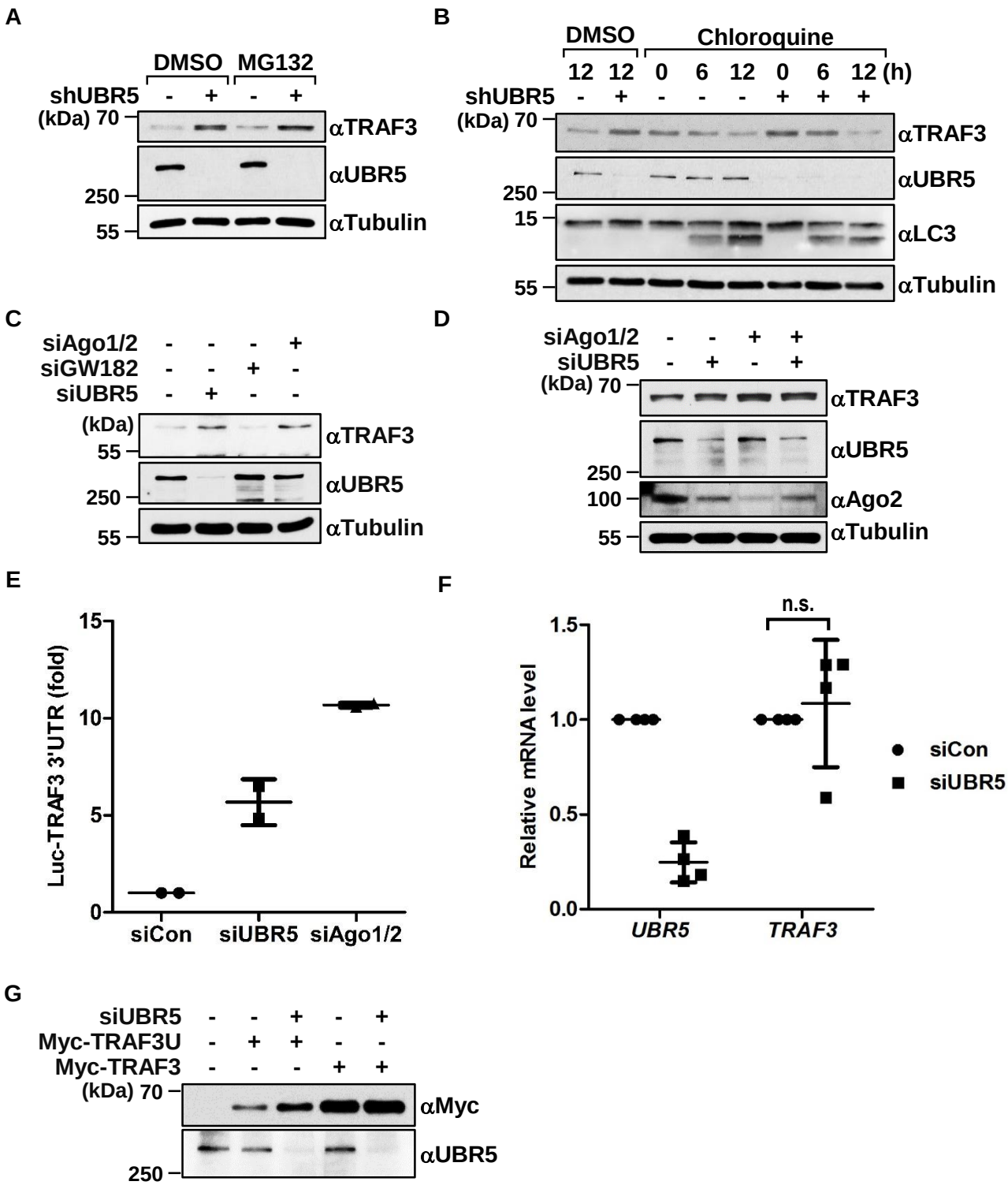
1.0
1.5
2.0
2.5
siCon
siUBR5

**Figure 1**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αTRAF3
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250
55
αTubulin
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DMSO
Chloroquine
12 12 0 6 12 0 6 12 (h)
shUBR5 - + - - - + + +
(kDa) 70
αTRAF3
250
αUBR5
15
αLC3
55
αTubulin
C
siAgo1/2 - - - +
siGW182 - - + -
siUBR5 - + - -
(kDa)
55
αTRAF3
250
αUBR5
55
αTubulin
D
siAgo1/2 - - + +
siUBR5 - + - +
(kDa) 70
αTRAF3
αUBR5
250
100
αAgo2
55
αTubulin
E
Luc-TRAF3 3'UTR (fold)
15
10
5
0
siCon
siUBR5
siAgo1/2
F
n.s.
Relative mRNA level
1.5
1.0
0.5
0.0
UBR5
TRAF3
siCon
siUBR5
G
siUBR5 - - + - +
Myc-TRAF3U - + + - -
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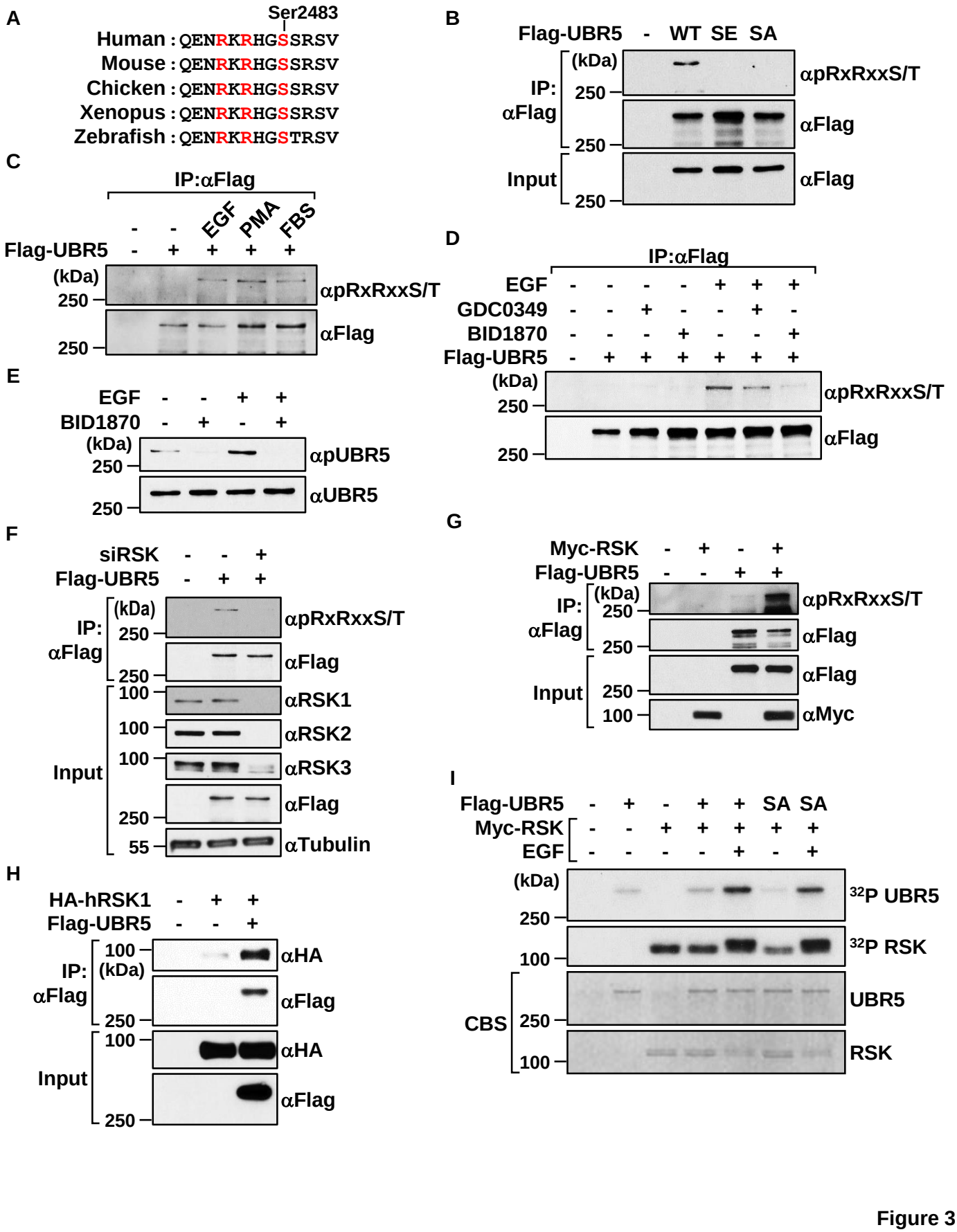
(kDa) 70
αMyc
αUBR5
250

Figure 2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(kDa)
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αFlag
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250
250
αpRxRxxS/T
αFlag
Input
100
100
100
250
55
αRSK1
αRSK2
αRSK3
αFlag
αTubulin
G
Myc-RSK - + - +
Flag-UBR5 - - + +
IP: αFlag
(kDa)
250
250
αpRxRxxS/T
αFlag
Input
250
100
αFlag
αMyc
H
HA-hRSK1 - + +
Flag-UBR5 - - +
IP: αFlag
100
(kDa)
250
αHA
αFlag
Input
100
250
αHA
αFlag
I
Flag-UBR5 - + - + + SA SA
Myc-RSK - - + + + + +
EGF - - - - + - +
(kDa)
250
100
CBS
250
100
32P UBR5
32P RSK
UBR5
RSK

Figure 3

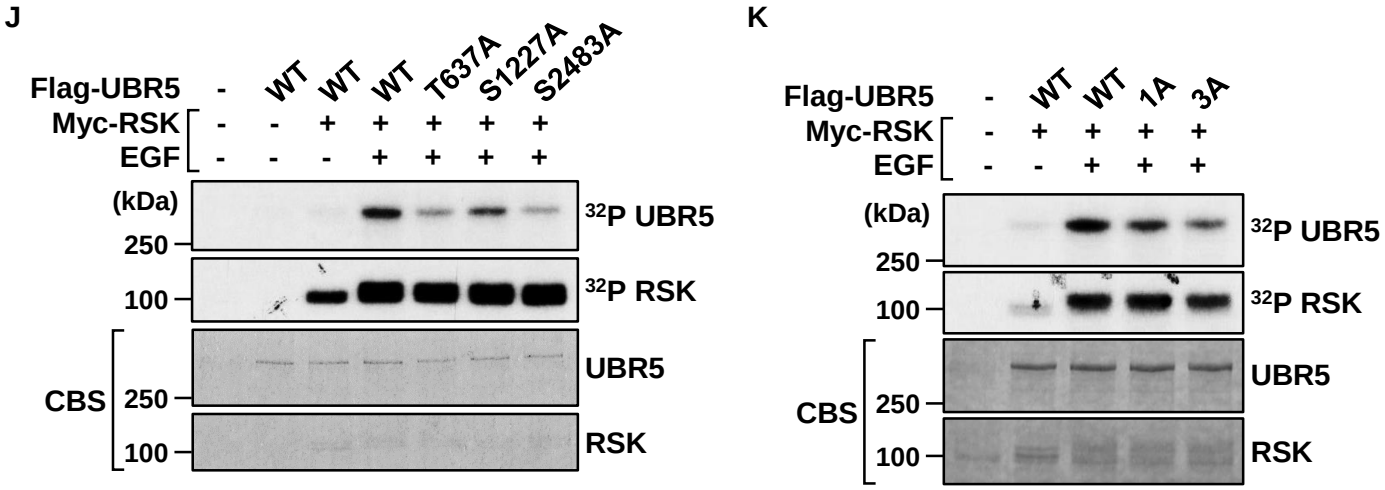
J
Flag-UBR5 - WT WT WT T637A S1227A S2483A
Myc-RSK - - + + + + +
EGF - - - + + + +
(kDa)
250
100
32P UBR5
32P RSK
CBS
250
100
UBR5
RSK
K
Flag-UBR5 - WT WT 1A 3A
Myc-RSK - + + + +
EGF - - + + +
(kDa)
250
100
32P UBR5
32P RSK
CBS
250
100
UBR5
RSK

**Figure 3**

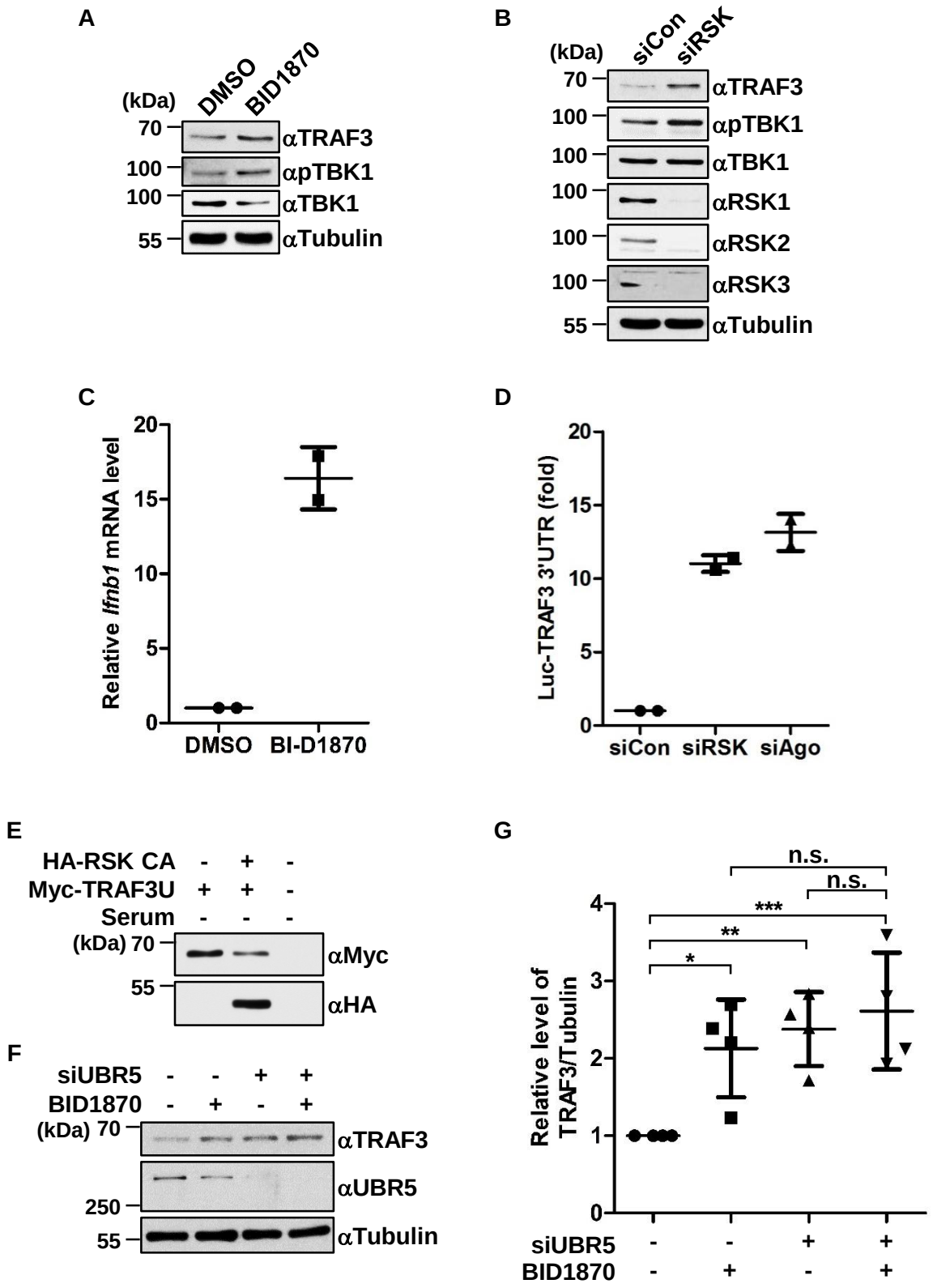
A
DMSO
BID1870
(kDa)
70
αTRAF3
100
αpTBK1
100
αTBK1
55
αTubulin
B
siCon
siRSK
(kDa)
70
αTRAF3
100
αpTBK1
100
αTBK1
100
αRSK1
100
αRSK2
100
αRSK3
55
αTubulin
C
Relative Ifnb1 mRNA level
20
15
10
5
0
DMSO
BI-D1870
D
Luc-TRAF3 3'UTR (fold)
20
15
10
5
0
siCon
siRSK
siAgo
E
HA-RSK CA - + -
Myc-TRAF3U + + -
Serum - - -
(kDa) 70
αMyc
55
αHA
F
siUBR5 - - + +
BID1870 - + - +
(kDa) 70
αTRAF3
αUBR5
250
55
αTubulin
G
n.s.
n.s.
***
**
*
Relative level of TRAF3/Tubulin
4
3
2
1
0
siUBR5 - - + +
BID1870 - + - +

Figure 4

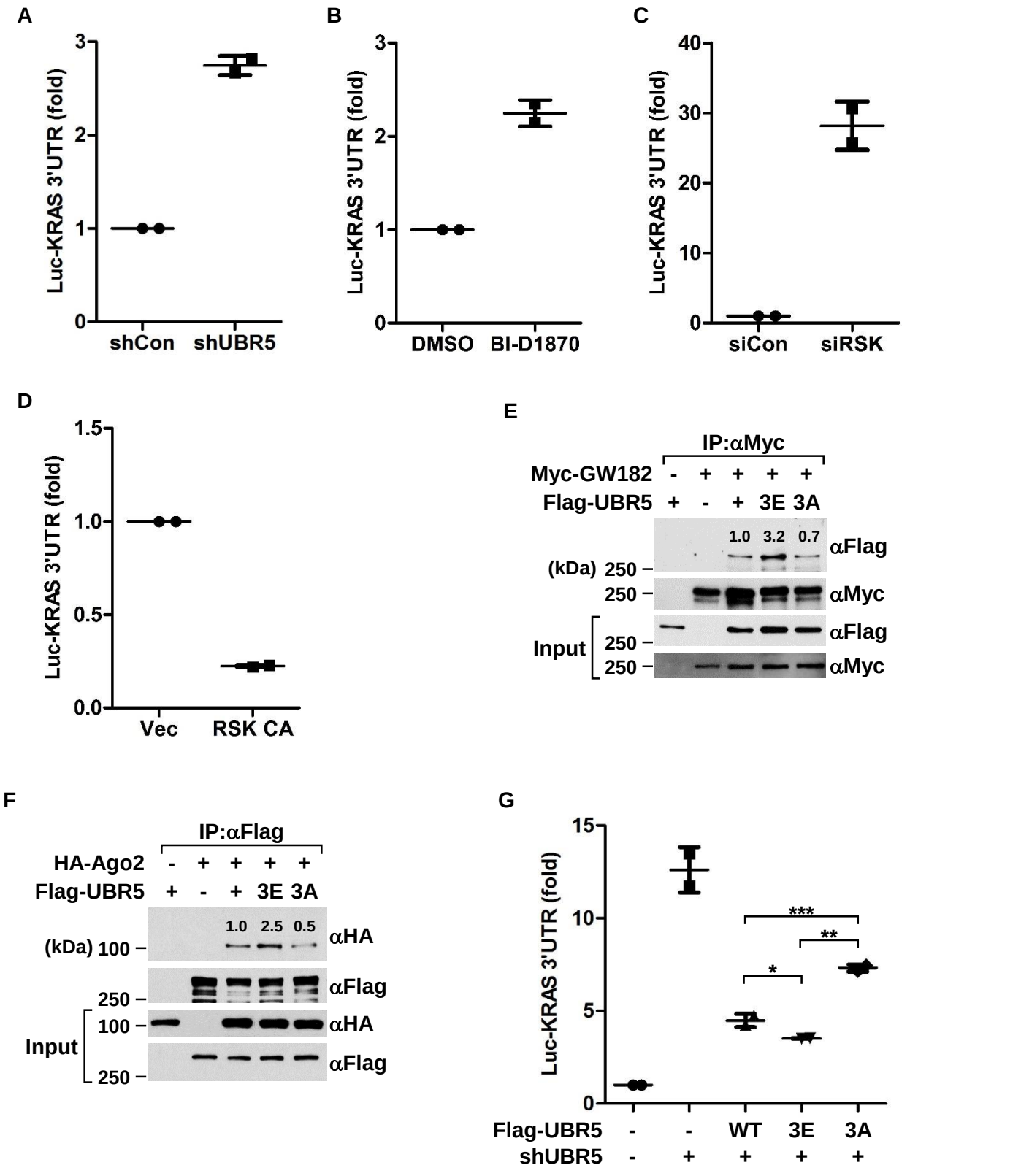
A
Luc-KRAS 3'UTR (fold)
shCon
shUBR5
B
DMSO
BI-D1870
C
siCon
siRSK
D
Vec
RSK CA
E
IP:αMyc
Myc-GW182
Flag-UBR5
3E
3A
1.0
3.2
0.7
(kDa)
αFlag
αMyc
Input
F
IP:αFlag
HA-Ago2
1.0
2.5
0.5
αHA
G
***
**
*
WT
shUBR5

Figure 5

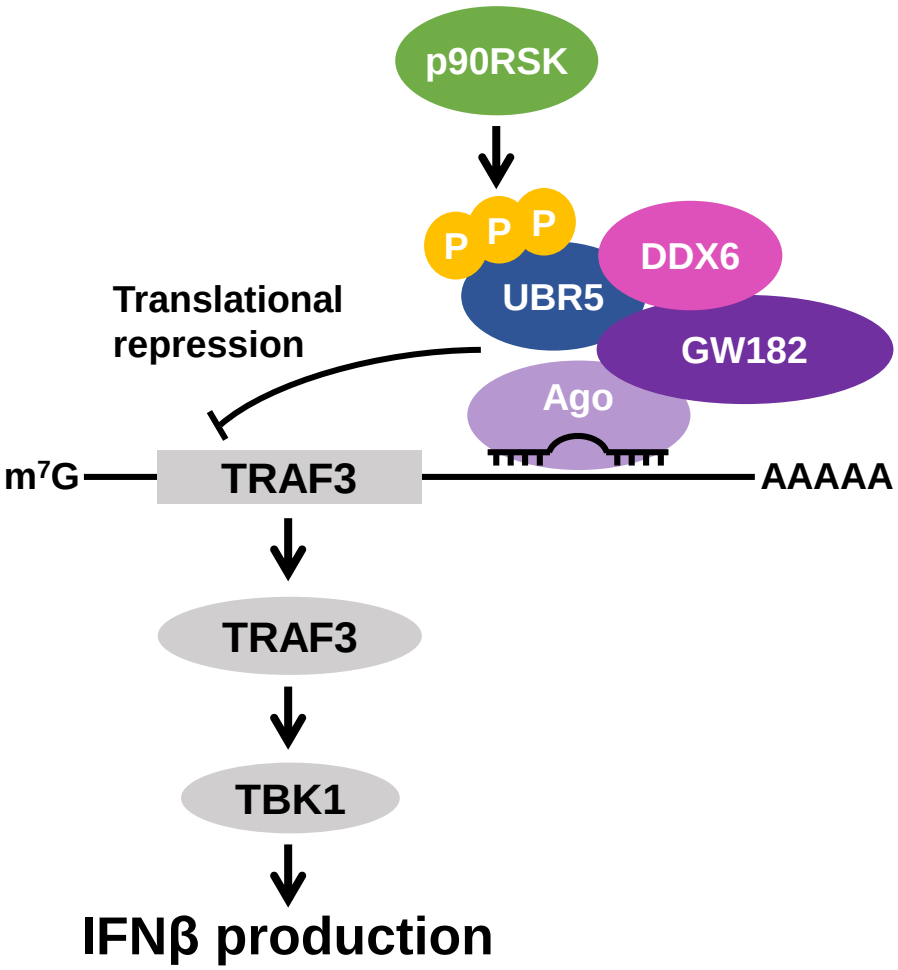
p90RSK
P
P
P
DDX6
UBR5
GW182
Translational
repression
Ago
m7G
TRAF3
AAAAA
TRAF3
TBK1
IFNβ production

Figure 6

**The p90 ribosomal S6 kinase–UBR5 pathway controls toll-like receptor signaling via miRNA–induced translational inhibition of TNF receptor–associated factor 3**

Jin Hwa Cho, Sung Ah Kim, Yeon-Soo Seo, Sung Goo Park, Byoung Chul Park, Jeong-Hoon Kim and Sunhong Kim

*J. Biol. Chem. published online May 30, 2017*

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