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hnRNPK inhibits GSK3 β Ser9 phosphorylation, thereby stabilizing c-FLIP and contributes to TRAIL resistance in H1299 lung adenocarcinoma cells

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c-FLIP (cellular FLICE-inhibitory protein) is the pivotal regulator of TRAIL resistance in cancer cells. It is a short-lived protein degraded through the ubiquitin/proteasome pathway. The discovery of factors and mechanisms regulating its protein stability is important for the comprehension of TRAIL resistance by tumor cells. In this study, we show that, when H1299 lung adenocarcinoma cells are treated with TRAIL, hnRNPK is translocated from nucleus to cytoplasm where it interacts and co-localizes with GSK3 β . We find that hnRNPK is able to inhibit the Ser9 phosphorylation of GSK3 β by PKC. This has the effect of activating GSK3 β and thereby stabilizing c-FLIP protein which contributes to the resistance to TRAIL in H1299 cells. Our immunohistochemical analysis using tissue microarray provides the clinical evidence of this finding by establishing a negative correlation between the level of hnRNPK expression and the Ser9 phosphorylation of GSK3 β in both lung adenocarcinoma tissues and normal tissues. Moreover, in all cancer tissues examined, hnRNPK was found in the cytoplasm whereas it is exclusively nuclear in the normal tissues. Our study sheds new insights on the molecular mechanisms governing the resistance to TRAIL in tumor cells, and provides new clues for the combinatorial chemotherapeutic interventions with TRAIL.

Lung cancer is the leading cause of cancer-related death in the world. Among all cases, more than 85% of them are non-small cell lung cancers (NSCLC)¹. NSCLC patients are usually inappropriate for surgical intervention and therefore require systemic chemotherapy and radiation therapy. However, very poor prognosis has been observed for the lung cancer patients due to the chemotherapy resistance. Development of effective therapeutic strategies aiming to overcome the drug resistance is therefore required to improve the prognosis and survival of lung cancer patients². During the past years, dealing with the chemotherapy resistance to the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) has become a subject of interest for the worldwide researchers^{3–6}. TRAIL is a promising therapeutic agent that selectively causes apoptosis in cancer cells while without toxicity toward normal human cells tested^{7,8}. Soluble TRAIL as well as agonistic antibodies against TRAIL-receptor are currently in clinical trials⁹. Meanwhile, approximately 50% of human cancer cell lines and most of human primary tumor cells have been reported to be resistant to TRAIL, which is the cause of the very limited therapeutic

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efficacy of the latter¹⁰. Hence, elucidating the molecular mechanisms of the resistance to TRAIL will help to find out the effective strategies for sensitizing cancer cells to TRAIL-induced apoptosis¹¹.

TRAIL is a member of the tumor necrosis factor (TNF) family, which induces apoptosis through binding to its death receptor TRAIL-R1 (DR4) and TRAIL-R2 (DR5), and activating the death receptor signaling pathways^{12,13}. After binding to TRAIL, its receptors oligomerize and recruit the cytoplasmic proteins FADD (Fas-associated death domain protein) and procaspase-8 (or procaspase-10) to form the death-inducing signaling complex (DISC)^{9,14}. The auto-activation of the caspase 8 in the complex results in the subsequent activation of effector caspases, including caspases 3, 6, and 7, and finally leads to cell apoptosis^{9,15}.

TRAIL-induced death receptor pathway is regulated by various factors. Among these factors, cellular FLICE-inhibitory protein (c-FLIP) is considered to be a master anti-apoptotic regulator and resistance factor^{16–18}. c-FLIP shares structural homology with procaspase-8 but does not contain the catalytic site as the latter. It can be therefore recruited to DISC through association with FADD to competitively inhibit the caspase 8 activation and acts as key suppressor of the death receptor signaling pathway^{16,19}. The increased expression of c-FLIP is detected in a wide range of cancers^{20,21}, and positively correlates with the resistance of cancer cells to death receptor ligands²². Conversely, the decreased expression of c-FLIP by chemicals or siRNA sensitizes cancer cells to death receptor-induced apoptosis^{16,22,23}. Both c-FLIP_L (55 kD) and p43 c-FLIP (43 kD, the caspase-8 processed N-terminal fragment of c-FLIP_L) could function as an apoptosis suppressor, with more efficiency of the latter^{24–27}.

The ubiquitous serine/threonine kinase Glycogen synthase kinase beta (GSK3 β) is another key regulator of apoptosis. GSK3 β is thought to facilitate the mitochondrial intrinsic apoptotic pathway while block death receptor-induced apoptosis²⁸. Inhibition or deletion of GSK3 β has been reported to sensitize death receptor-induced apoptosis in numerous tumor cells^{29–32}. Notably, inhibition of GSK3 by Celecoxib promoted the degradation of c-FLIP and death receptor-induced apoptosis, suggesting that GSK3 might stabilize c-FLIP and antagonized tumor resistance to TRAIL³³.

We have previously identified hnRNPk (heterogeneous nuclear ribonucleoprotein K) as a putative interacting partner of GSK3 β ³⁴. hnRNPk is a well conserved DNA and RNA binding protein and shares with several other RNPs the triple K-homology domain. hnRNPk shuttles between nucleus and cytoplasm and regulates gene expression at multiple levels^{35,36}. The expression of hnRNPk is aberrantly increased in a variety of cancers^{37–40}, and it has been reported that hnRNPk negatively regulated the TRAIL-induced apoptosis through up-regulating the transcriptional level of c-FLIP⁴¹.

The physical interaction between two antagonists of TRAIL-induced apoptosis: hnRNPk and GSK3 β , together with their respective functional interactions with c-FLIP have prompted us to investigate the functional relationship among these three proteins in the resistance of TRAIL-induced apoptosis. In this study, we focused on the possible role of hnRNPk-GSK3 β interaction in the regulation of the protein stability of c-FLIP, as well as the related functional consequence on the resistance to TRAIL-induced apoptosis of lung cancer cells.

Results

Interaction and co-localization of GSK3 β with hnRNPk in H1299 lung adenocarcinoma cells.

We have previously demonstrated that GSK3 β interacts with hnRNPk in HepG2 hepatocellular carcinoma cells³⁴. Here, we firstly used GST pull-down assays to demonstrate this interaction using the cellular lysate of lung adenocarcinoma cancer H1299 cells. As shown in Fig. 1a, purified GST-hnRNPk was capable to specifically pull down the endogenous GSK3 β in H1299 cell lysate. Interestingly, both anti-GSK3 β antibody and anti-p9-GSK3 β antibody were able to reveal the GSK3 β trapped by the purified GST-hnRNPk. This raised the uncertainty about the phosphorylation state of the trapped GSK3 β . Indeed, the GSK3 β molecules interacting with GST-hnRNPk could be both Ser9 phosphorylated and unphosphorylated, or they could be only Ser9 phosphorylated and recognized by both the anti-GSK3 β and anti-p9-GSK3 β antibodies. These two different situations might respectively reflect the two possibilities where the Ser9 phosphorylated and unphosphorylated GSK3 β interact equally or differentially with hnRNPk. To clarify this point, we have performed GST-pull down assays using H1299 cell lysates incubated respectively with either GST-GSK3 β *wt* or GST-GSK3 β S9A, a Ser9-phospho-defective mutant, and compared the quantity of hnRNPk trapped by these two forms of GSK3 β . Our results shown in Fig. S1 indicate that GST-GSK3 β *wt* and GST-GSK3 β S9A displayed similar affinities vis-à-vis hnRNPk in H1299 cell lysates. Therefore, Ser9 phosphorylation *per se* might not be a factor directly affecting the affinity between GST-GSK3 β and hnRNPk. The interaction between GSK3 β and hnRNPk was then confirmed by co-immunoprecipitation experiments using the H1299 cells transfected by Flag-hnRNPk. As shown in Fig. 1b, Flag-hnRNPk co-immunoprecipitated with GSK3 β by anti-GSK3 β antibody in the cell lysate of H1299 cells either treated or not by TRAIL. Confocal immunofluorescence assays were then performed to confirm this result by showing the co-localization of GSK3 β and hnRNPk in the cytoplasm of H1299 cells. Figure 1c,d demonstrate that both endogenous and ectopically expressed hnRNPk could be partially translocated from nucleus to cytoplasm upon TRAIL treatment, and co-localized with GSK3 β that was mainly cytoplasmic.

Both GSK3 β and hnRNPk antagonized TRAIL-induced apoptosis. In order to investigate the functional importance of GSK3-hnRNPk interaction in the TRAIL-induced apoptosis of H1299 cells, we searched at first to determine the optimal concentration and timing of TRAIL treatment on H1299 cells. A TRAIL dose-response experiment has been performed. H1299 cells were treated during 24 hours with increasing doses of TRAIL, and then analyzed by MTT assays for cellular viability and by Western blotting for the level of cleaved Caspase 3, both indicators of the apoptotic degree of cells. Our results in Fig. 2a,b showed that, when used within the range of 2–20 ng/ml, TRAIL induced apoptosis of H1299 cells in a dose-dependent manner, with a marked effect observed at 20 ng/ml, as judged by both the cleaved caspase 3 level and the cell death rate. This concentration was then used for another experiment destined to determine the timing of the treatment. Cells were harvested at different time points after TRAIL stimulation and analyzed as above for the apoptotic state of the cells.

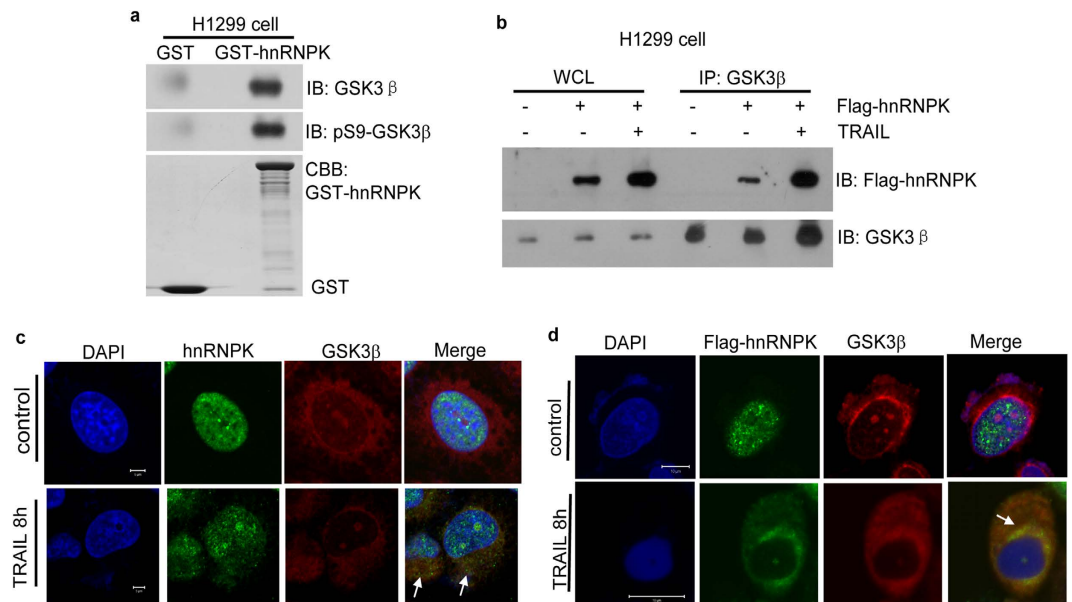


Figure 1. Interaction and co-localization of GSK3 β with hnRNPK in H1299 cells. (a) GST-hnRNPK pull-down assay. H1299 cell lysates were incubated with GST or GST-hnRNPK fusion proteins immobilized on glutathione sepharose beads as described. Western blotting was then performed with GSK3 β and phospho-GSK3 β (Ser9) antibodies to detect the presence of GSK3 β and phospho-GSK3 β (Ser9) in the bound fractions. The amounts of recombinant proteins GST and GST-hnRNPK were estimated by CBB staining. (b) Co-immunoprecipitation assay of the interaction of GSK3 β with hnRNPK. H1299 cells were transfected with Flag-hnRNPK or empty Flag-vector as indicated and treated with TRAIL (20 ng/ml, 8 hours). The existence of Flag-hnRNPK and GSK3 β in the Sepharose-beads bound fractions were analyzed by immunoblot (IB) with Flag and GSK3 β antibodies. WCL: whole cell lysates. (c) Subcellular localization of GSK3 β and hnRNPK in H1299 cells after TRAIL treatment. H1299 cells treated with or without TRAIL (20 ng/ml, 8 hours) were fixed and stained with both GSK3 β and hnRNPK antibodies. The Alexa Fluor 488 and Alexa Fluor 594 conjugated secondary antibody were used. Arrows: the co-localization of the two proteins. Bar: 5 μ m. (d) Subcellular localization of GSK3 β and ectopically expressed hnRNPK in H1299 cells after TRAIL treatment. H1299 cells transfected with Flag-hnRNPK were treated with TRAIL (20 ng/ml, 8 hours), then fixed and stained with both GSK3 β and Flag antibodies. The same secondary antibodies used in (c) were used. Arrows: the co-localization of the two proteins. Bar: 10 μ m.

Our experiment suggested that 8–12 hours of TRAIL treatment were sufficient to induce significant apoptosis in H1299 cells (Fig. 2c,d). Subsequently, we attempted to assess the role of GSK3 β in the TRAIL-induced apoptosis of H1299 cells by using LiCl that is a specific inhibitor of GSK3 β kinase activity and inducer of the Ser9 phosphorylation of GSK3 β ⁴². In our experiment, increasing doses of LiCl, ranging from 5 mM to 50 mM, indeed resulted in the increasing level of GSK3 β Ser9 phosphorylation, which was also confirmed by the increasing level of β -catenin whose degradation is an indicator of the kinase activity of GSK3 β (Fig. 2e). The concentration of 20 mM was then chosen for the subsequent experiments of LiCl treatment. As described in Materials and Methods, we have treated H1299 cells separately or jointly with 20 mM LiCl and 20 ng/ml TRAIL, then examined the level of total GSK3 β , Ser9 phosphorylated GSK3 β , and cleaved Caspase 3 in these cells by Western blotting using specific antibodies (Fig. 2f). Our experiments showed that LiCl was capable to enhance the effect of TRAIL to induce apoptosis, reflected by the increased level of cleaved Caspase 3. MTT assays were then performed with This preliminary result conforming the pro-survival role of active GSK3 β under the treatment of TRAIL was consistent with other reports^{31,43}.

To further investigate the functions of both GSK3 β and hnRNPK in TRAIL-induced tumor cell apoptosis, we have either knocked down or overexpressed GSK3 β or hnRNPK respectively by transient transfection of H1299 cells with corresponding siRNA or Flag-tagged expression plasmids. Cells were then examined by Western blotting using specific antibodies against Flag tag, GSK3 β , hnRNPK and cleaved Caspase 3, or by FACS with Annexin V/PI double staining (Fig. 3a–h). As shown, knockdown of either GSK3 β or hnRNPK significantly increased the level of the cleaved Caspase 3 in Western blotting analyses and also the cell apoptotic rates in FACS assays, whereas the opposing effects were observed when cells were transfected by Flag-GSK3 β or Flag-hnRNPK (Fig. 3a–h). These results indicated that both GSK3 β and hnRNPK antagonized TRAIL-induced apoptosis in H1299 cells.

hnRNPK down-regulates the Ser9 phosphorylation of GSK3 β by PKC in H1299 cells. In our preliminary studies, we have observed that the overexpression of Flag-hnRNPK dramatically reduced the Ser9 phosphorylation of GSK3 β in several kinds of cells, including H1299 cells (data not shown). This suggests that hnRNPK might inhibit the Ser9 phosphorylation of GSK3 β and thereby regulate the TRAIL-induced apoptosis in

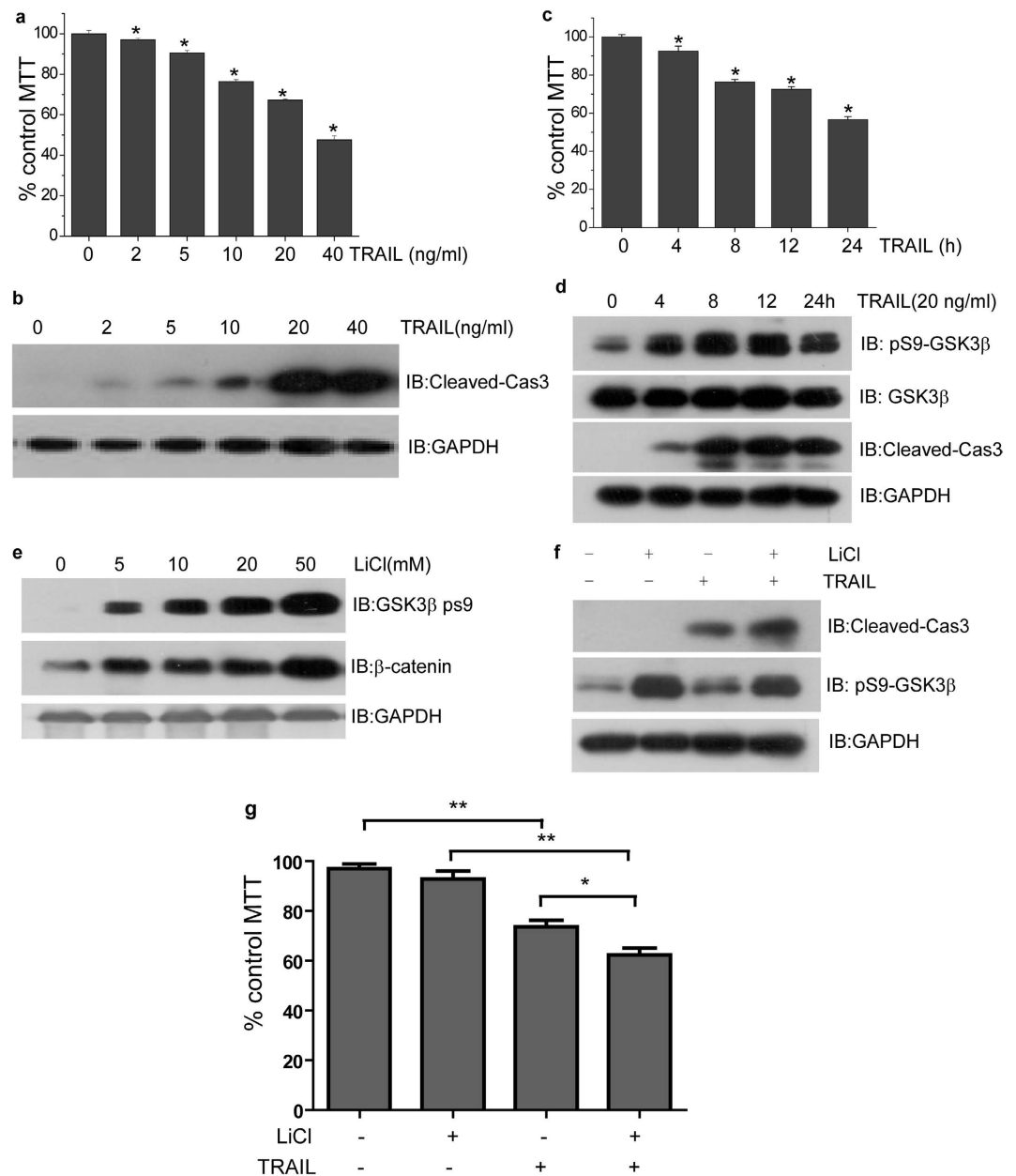


Figure 2. Both GSK3β and hnRNPK antagonized TRAIL-induced apoptosis. (a) MTT assays of cellular viability with TRAIL treatment at different concentrations. H1299 cells were seeded into 96-well plate (4000 cells per well) and treated with TRAIL at increasing concentrations as indicated for 24 hours. MTT assays were then performed as described in Method section. The cellular viability was monitored by the absorbance of formazan crystals at 570 nm. The absorbance of the control group was set as 100%. * $p < 0.05$ (compared with control group). (b) Cleaved-Cas3 level in H1299 cells treated with TRAIL at various concentrations. H1299 cells were treated with TRAIL within the range of 2–20 ng/ml for 24 hours and harvested for Western blot analysis using cleaved-Cas3 antibody. (c) MTT assays of cellular viability after TRAIL treatment at different timescales as indicated. H1299 cells were treated with TRAIL at concentrations of 20 ng/ml for 4–24 hours, then analyzed with MTT using the same methods with (a). * $p < 0.05$ (compared with control group). (d) Cleaved-Cas3 level in H1299 cells treated with TRAIL during different timescales. H1299 cells were treated with TRAIL at concentrations of 20 ng/ml for 4–24 hours and analyzed by Western blotting using cleaved-Cas3 antibody. (e) The effect of LiCl on the activity of GSK3β in H1299 cells. Cells were treated with LiCl at various concentrations as indicated for 8 hours and harvested for Western blotting analysis with the indicated antibodies. (f,g) LiCl enhanced the apoptotic effect of TRAIL. H1299 cells treated with either LiCl (20 mM, 8 hours) or TRAIL (20 ng/ml, 8 hours) alone, or the combination of LiCl and TRAIL, were analyzed by Western blotting with the indicated antibodies (f), or by MTT assays (g). * $p < 0.05$. ** $p < 0.01$.

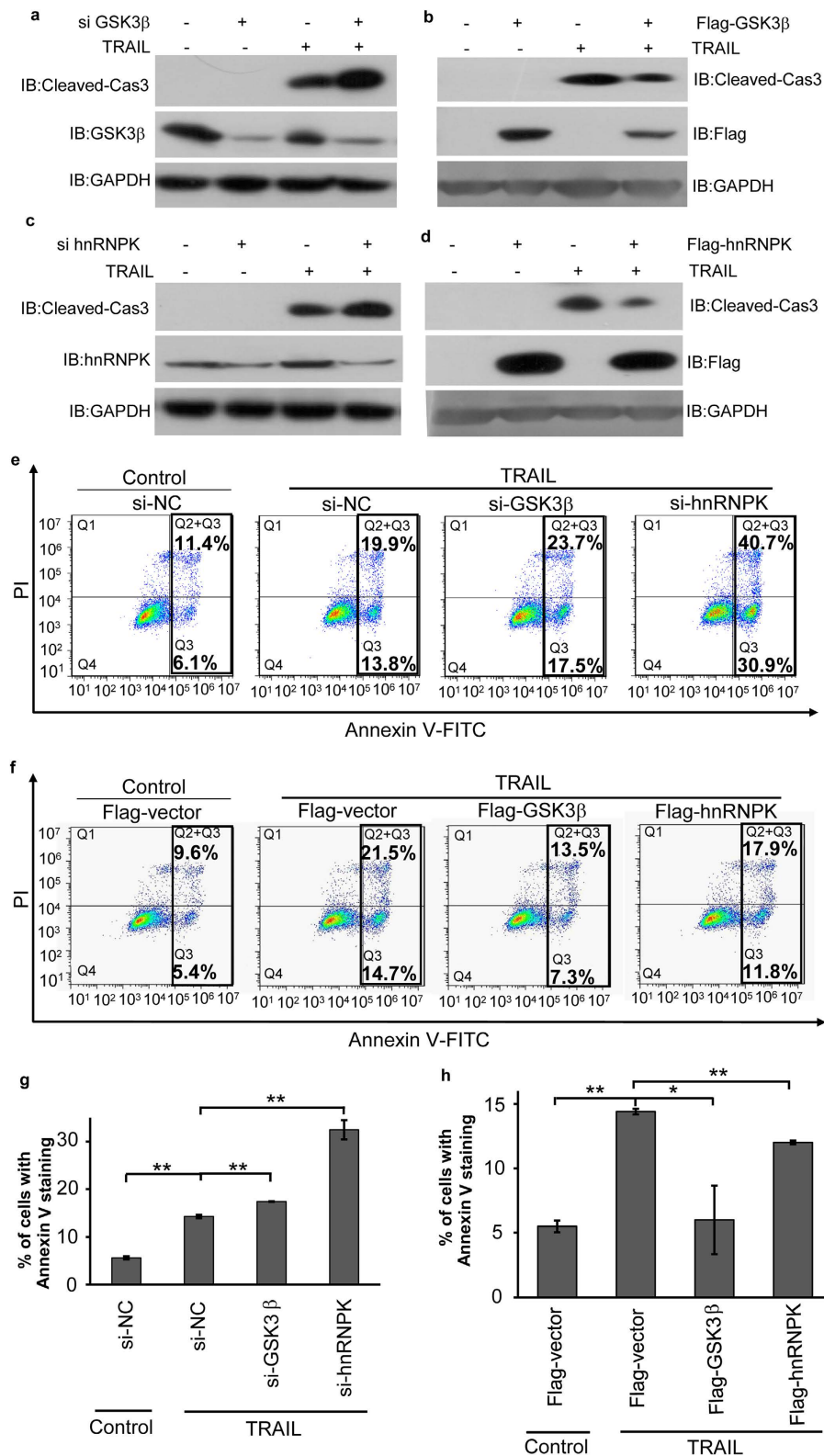


Figure 3. Both GSK3β and hnRNPk antagonized TRAIL-induced apoptosis. H1299 cells were transfected with siRNA against GSK3β (a), Flag-GSK3β (b), siRNA against hnRNPk (c), or Flag-hnRNPk (d), and treated with TRAIL (20 ng/ml, 8 hours), then subjected to Western blot analysis with the indicated antibodies. (e,f) Analysis of the cell death rate by Flow cytometry. H1299 cells transfected with GSK3β siRNA or hnRNPk siRNA (e), or transfected with Flag-GSK3β or Flag-hnRNPk (f) were treated with TRAIL (20 ng/ml, 8 hours) and analyzed using Annexin V/PI double staining with a FACS Calibure flow cytometer. (g,h) Statistical analyses of the experiments shown in (e,f) respectively. The analyses were performed with the results of three independent replicates for each experiment. *p < 0.05.

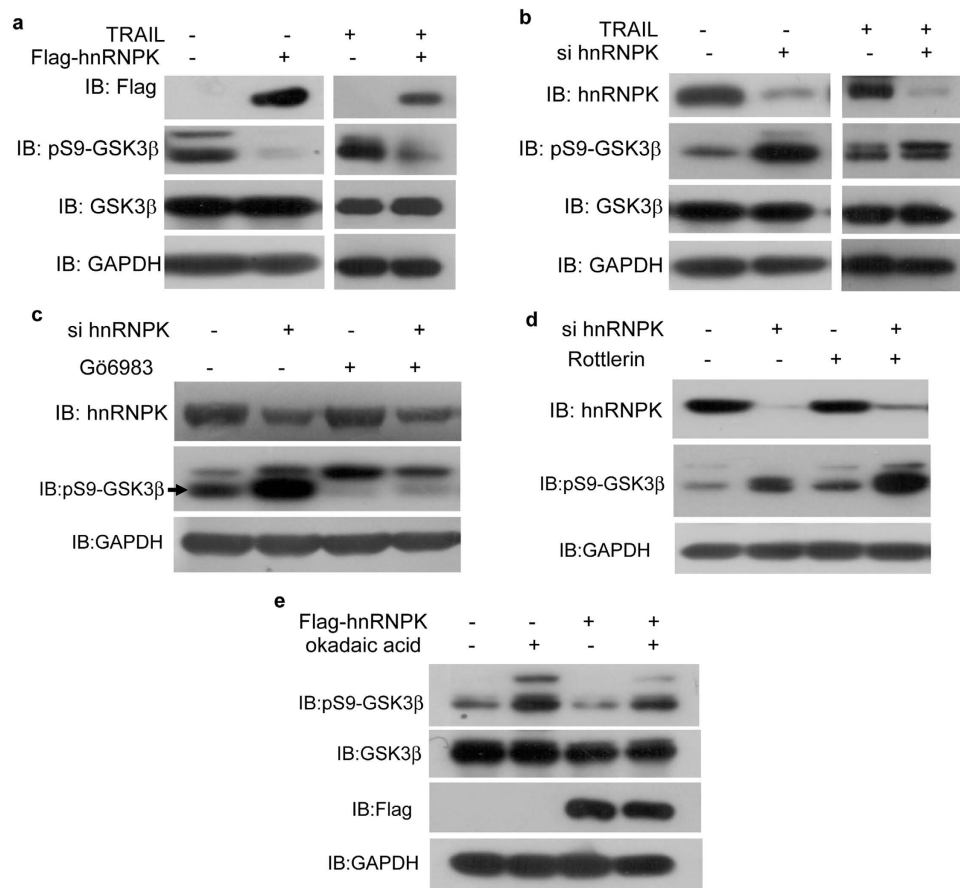


Figure 4. hnRNPK inhibited GSK3β Ser9 phosphorylation by PKC. H1299 cells respectively transfected with Flag-hnRNPK (a) or hnRNPK siRNA (b), and treated with or without TRAIL (20 ng/ml, 8 hours) were harvested for Western blot analysis with the indicated antibodies. (c) PKC inhibitor Gö6983 cancelled the regulatory effect of hnRNPK on GSK3β Ser9 phosphorylation. H1299 cells transfected with hnRNPK siRNA or/and treated with Gö6983 (1 μM) were analyzed by Western blotting with the indicated antibodies. (d) PKC inhibitor Rottlerin had no obvious effect on the regulation of GSK3β Ser9 phosphorylation by hnRNPK. H1299 cells transfected with hnRNPK siRNA or/and treated with Rottlerin (6 μM) were analyzed by Western blotting with the indicated antibodies. (e) PP1 inhibitor Okadaic acid did not affect the regulation of GSK3β Ser9 phosphorylation by hnRNPK. H1299 cells transfected with Flag-hnRNPK or/and treated with okadaic acid (100 nM) were analyzed by Western blotting with the indicated antibodies.

H1299 cells. To confirm this hypothesis, we have either overexpressed Flag-hnRNPK or knocked down hnRNPK by siRNA in H1299 cells with or without TRAIL treatment, and examined the levels of total GSK3β and Ser9 phosphorylated GSK3β. As shown in Fig. 4a,b, the overexpression and depletion of hnRNPK respectively resulted in sharp inhibition and stimulation of GSK3β Ser9 phosphorylation, regardless the cells were treated or not with TRAIL, while the level of total GSK3β remained unaffected. This result clearly indicates that hnRNPK is capable of down-regulating the Ser9 phosphorylation of GSK3β.

Various serine/threonine kinases have been shown to be able to phosphorylate GSK3β on its serine 9 residue, including PKA, PKB/Akt, PKC, p70^{S6K}, p90^{RSK}/MAPKAP kinase-1^{44–46}. Notably, PKC kinase has been reported to phosphorylate GSK3^{33,47–49} and positively regulate celecoxib-induced NSCLC cell apoptosis involving the activation of the death receptor pathway³³. Therefore, we investigated if hnRNPK could inhibit the Ser9 phosphorylation of GSK3β by PKC in H1299 cells.

Firstly, we confirmed that the Ser9 phosphorylation of GSK3β in H1299 cells was mainly the effect of PKC kinase activity, since Gö6983, an inhibitor against the classic PKC α-ξ subtypes, was capable to inhibit the quasi totality of Ser9 phosphorylation of GSK3β in these cells (Fig. 4c). And more interestingly, with Gö6983 treatment, the knocking down of hnRNPK by siRNA had no longer obvious effect on the Ser9 phosphorylation of GSK3β. As contrast, when using rottlerin that is an inhibitor against the non-classic PKC δ subtype, hnRNPK siRNA still significantly stimulated the Ser9 phosphorylation of GSK3β (Fig. 4d). These results suggest that hnRNPK inhibits the Ser9 phosphorylation of GSK3β by PKC α-ξ but not PKC δ in H1299 cells. However, since the Ser9 phosphorylation state of GSK3β was regulated both by its phosphorylation by PKC and its dephosphorylation by PP1⁵⁰, it remained to verify if hnRNPK might also stimulate the dephosphorylation of GSK3β by PP1. To address this issue, we have treated H1299 cells with 100 nM okadaic acid which inhibited the dephosphorylation of GSK3β by

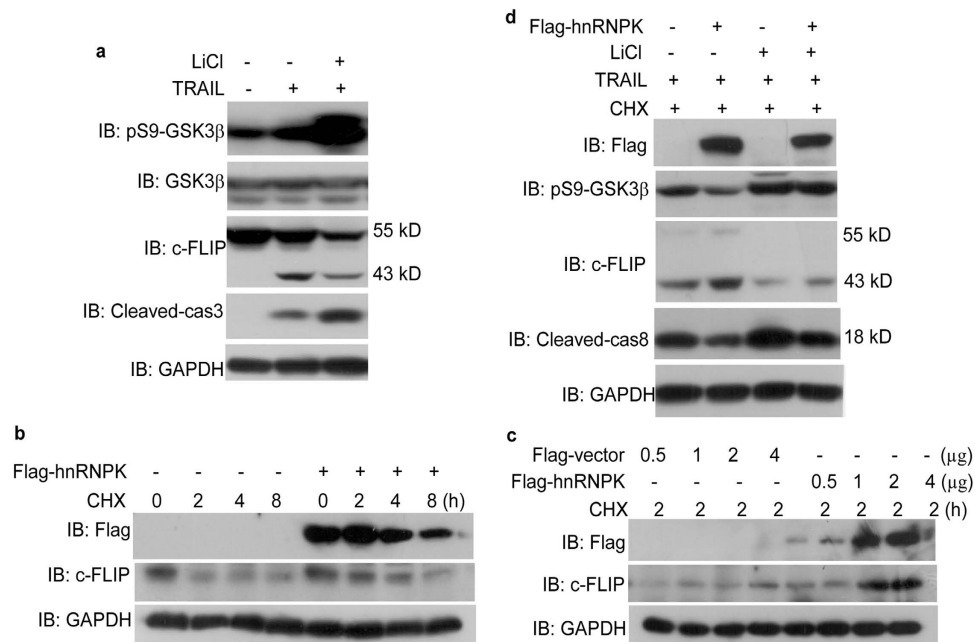


Figure 5. hnRNPK stabilized of c-FLIP protein through inhibition of GSK3β Ser9 phosphorylation during the TRAIL-induced apoptosis. (a) GSK3β upregulated the protein level of c-FLIP in H1299 cells treated with TRAIL. H1299 cells treated with LiCl (20 mM, 8 hours) and/or TRAIL (20 ng/ml, 8 hours) were harvested for Western blot analysis with the indicated antibodies. (b) Effect of hnRNPK overexpression on c-FLIP protein stability by Cycloheximide (CHX) chase experiments. H1299 cells transfected with either 2 μg Flag-hnRNPK or Flag-vector plasmids were treated with 10 μg/ml CHX for the indicated durations, then subjected to Western blot analysis with the indicated antibodies. (c) Effect of hnRNPK overexpression on c-FLIP protein stability by dose-response experiment. H1299 cells were transfected with Flag-hnRNPK or Flag-vector plasmids at the indicated doses and stimulated with CHX (10 μg/ml, 2 h), then subjected to Western blot analysis with the indicated antibodies. (d) Effect of hnRNPK on c-FLIP protein stability was dependent on the Ser9 phosphorylation state of GSK3β. H1299 cells transfected with Flag-hnRNPK or Flag-vector plasmids were stimulated with CHX (10 μg/ml, 2 h), TRAIL (20 ng/ml, 8 hours), or LiCl (20 mM, 8 hours) as indicated, then subjected to Western blot analysis with the indicated antibodies.

PP1, and examined the effect of hnRNPK expression on the phosphorylation state of GSK3β. Our result showed that the treatment by okadaic acid again did not alter the inhibitory effect of hnRNPK overexpression, excluding the possible regulation of GSK3β dephosphorylation by hnRNPK (Fig. 4e).

Taken together, these results suggest that hnRNPK is capable to inhibit the Ser9 phosphorylation of GSK3β by PKC, probably via steric hindrance caused by its interaction with GSK3β.

hnRNPK inhibits GSK3β Ser9 phosphorylation, leading to the stabilization of c-FLIP protein and inactivation of Caspase 8 during the TRAIL-induced apoptosis of H1299 cells. Based on the regulation of GSK3β Ser9 phosphorylation by hnRNPK as described hereinabove, and the stabilization of c-FLIP by GSK3β reported by others³³, we searched to know if there was any link between these two events which might be part of the mechanisms involving these three proteins in the resistance of the TRAIL-induced apoptosis. We have firstly confirmed the regulatory role of GSK3β on the protein level of c-FLIP in H1299 cells treated with TRAIL. As shown in Fig. 5a, LiCl treatment of cells resulted in the increased level of GSK3β Ser9 phosphorylation and the down-regulation of both p55 and p43 c-FLIP protein levels, and as a consequence, the augmentation of the cleaved Caspase 3 amount. Next, we examined the effect of hnRNPK overexpression on c-FLIP protein stability in H1299 cells by performing cycloheximide chase experiments. H1299 cells transfected by either 2 μg Flag-hnRNPK or empty Flag plasmids were treated with 10 μg/ml cycloheximide (CHX). After various durations of treatment, cells were harvested and subjected to Western blotting analysis to assess the protein level of c-FLIP. Our results in Fig. 5b shows that in cells transfected with the empty Flag vector, most of the c-FLIP protein is degraded as rapidly as 2 hours after the treatment with CHX, while the overexpression of Flag-hnRNPK significantly slows down the c-FLIP degradation (Fig. 5b). The stabilizing effect of hnRNPK on c-FLIP protein was then further confirmed by a dose-response experiment. Cells transfected by increasing amount of either Flag-hnRNPK or empty Flag plasmids and treated with 10 μg/ml CHX during 2 hours were harvested. The protein level of c-FLIP was then assessed by Western blotting as described. As shown in Fig. 5c, the increasing concentration of overexpressed Flag-hnRNPK indeed resulted in the augmentation of c-FLIP in the cells as compared with the empty vector-transfected cells. And, as expected, the effect of hnRNPK was dependent on the Ser9 phosphorylation state of GSK3β, since LiCl treatment of the cells cancelled this effect. Moreover, c-FLIP expression levels are inversely correlated with those of the cleaved caspase 8, further confirming the functional effectiveness of this regulatory

pathway (Fig. 5d). These results suggest that, in addition to the possible modulation of c-FLIP expression by hnRNPK at the transcriptional level as described in other report⁴¹, hnRNPK might also stabilize c-FLIP protein through regulation of GSK3 β Ser9 phosphorylation.

hnRNPK expression negatively correlates with Ser9 phosphorylated GSK3 β in clinical lung adenocarcinoma tissues.

In order to provide the clinical evidence of the regulation of GSK3 β Ser9 phosphorylation by hnRNPK in lung cancer, we have performed the immunohistochemical essays using two commercialized tissue microarrays. 52 paired lung adenocarcinoma and adjacent normal samples were stained with either anti-hnRNPK antibody or anti-Ser9 phosphorylated GSK3 β antibody, evaluated and scored as described in Materials and methods section. The results showed that hnRNPK was highly overexpressed in both the nucleus and cytoplasm of lung adenocarcinoma cells as compared with normal tissues (Fig. 6a–c), whereas the phospho-GSK3 β was mainly located in the cytoplasm which was significantly downregulated in the tumor tissues (Fig. 6d). Furthermore, negative correlation between the level of hnRNPK and the Ser9 phosphorylated GSK3 β could be established in both lung cancer and normal tissues (Fig. 6e). Strikingly, in the same histological section of lung cancer, we were able to simultaneously observe the low hnRNPK expression accompanied with the high level of phospho-GSK3 β in a region (the arrows, Fig. 6e), and the opposite pattern in a neighbouring zone (the stars, Fig. 6e). Statistical analysis demonstrated that the expression levels of hnRNPK and phospho-GSK3 β were inversely correlated in lung tumor tissues ($p < 0.05$, two-tailed). Finally, we found that in 52 (100%) carcinoma tissues examined, hnRNPK could be found in both the cytoplasm and nucleus whereas it was exclusively nuclear in normal tissues (Fig. 6f). This is consistent with another study performed by Pino and colleagues showing the cytoplasmic staining of hnRNPK in half of the 32 lung carcinoma samples examined⁵¹. This result strongly suggests the importance of the cytoplasmic hnRNPK in the genesis of lung adenocarcinoma, and its role in the regulation of the GSK3 β Ser9-phosphorylation.

Discussion

Owing to its capacity to specifically induce the apoptosis in tumor cells but not in normal cells, TRAIL is predicted to be one of the most effective anticancer therapeutic agents in the future^{7,8,52}. However, resistance to TRAIL has turned out to be a general phenomenon and become a serious obstacle for the prospects of TRAIL therapy. The comprehensive understanding of the molecular mechanisms of TRAIL's action thus reveals to be important and necessary for the development of combinatorial treatments involving TRAIL and others synergizing or sensitizing agents. Previous studies have established that the effects of TRAIL pass through the membrane death receptors 4 and 5 (DR4 and DR5) with their intracellular death domains, which activates dual opposing pathways leading respectively to the apoptosis but also the survival of the cell⁵². These two so called "apoptotic pathway" and "resistance pathway" are thought to be respectively responsible for the apoptotic effect of TRAIL and resistance to TRAIL in the cells. c-FLIP is regarded as the master anti-apoptotic regulator and resistance factor in TRAIL-induced apoptosis due to its effect of shifting the apoptotic pathway to the resistance pathway by facilitating the formation of the so called "secondary complex". The observation that chemotherapeutic agents like celecoxib, camptothecin, and cisplatin could down regulate c-FLIP expression and sensitize the resistant cells to TRAIL treatment further demonstrated that c-FLIP plays a key role in the resistance to TRAIL-induced apoptosis and represents a potential target candidate for the combinatorial chemotherapeutic interventions with TRAIL^{33,53}. c-FLIP has been shown to be a short-lived protein that is degraded via the proteasome pathway^{17,23,54}. Therefore, the regulation of its stability might represent an interesting way to overcome the resistance to TRAIL. Our present study describes a novel mechanism of action of hnRNPK to antagonize TRAIL-induced apoptosis. hnRNPK is a DNA/RNA binding protein whose function has been so far associated with its nuclear localization and/or its regulation of the RNA expression and metabolism. We show here for the first time that the cytoplasmic hnRNPK interacts with GSK3 β , and regulates its function to stabilize c-FLIP protein. Our finding is corroborated by our immunohistochemical analysis with tissue microarray demonstrating the negative correlation between hnRNPK expression level and that of the Ser9-phosphorylated GSK3 β in lung adenocarcinoma tissues. Interestingly, hnRNPK has been previously reported to transcriptionally activate c-FLIP and antagonize TRAIL-induced apoptosis in nasopharyngeal carcinoma cells⁴¹. Taken together, these studies suggest that hnRNPK may up-regulate the c-FLIP protein level in cancer cells through two different mechanisms and at two different subcellular locations to antagonize the apoptotic effect of TRAIL, which would make this protein an interesting target for the combinatorial therapy with TRAIL.

GSK3 β is a ubiquitous serine/threonine kinase with multiple functions involved in a wide range of cellular functions, including differentiation, growth, apoptosis, cell cycle, embryonic development, and insulin response^{55–57}. The phosphorylation on the serine 9 residue of GSK3 β is critical for its multiple functions. Our present study has permitted to identify hnRNPK protein as an efficient activator of GSK3 β through inhibiting its Ser9 phosphorylation, one of the most active and versatile family of kinases responding to a variety of extracellular signals to regulate multiple signaling pathways in many types of cells⁵⁸. Thus, in addition to its classic role of a DNA/RNA binding protein involved in the metabolism and expression of RNA, the discovery of its role as a regulator of GSK3 β playing key roles in many signaling pathways and cellular processes turns out to be very exciting. The investigation of the possible regulation of GSK3 β activity by hnRNPK in various physiological and pathological contexts reveals to be, in this regard, very interesting and important.

How could hnRNPK inhibit the phosphorylation state of GSK3 β ? Two possibilities could be taken into consideration. hnRNPK might either inhibit the phosphorylating activity of PKC, or facilitate the action of PP1 which dephosphorylates Ser9 phosphorylated GSK3 β . The results of our experiments using the inhibitors respectively against PKC or PP1 have shown that hnRNPK acts more likely through the regulation of PKC rather than that of PP1 (Fig. 4c,e). The most probable scenario should be the stoichiometric hindrance caused by the hnRNPK-GSK3 β interaction which renders inaccessible the Ser9 residue to PKC. We have attempted to

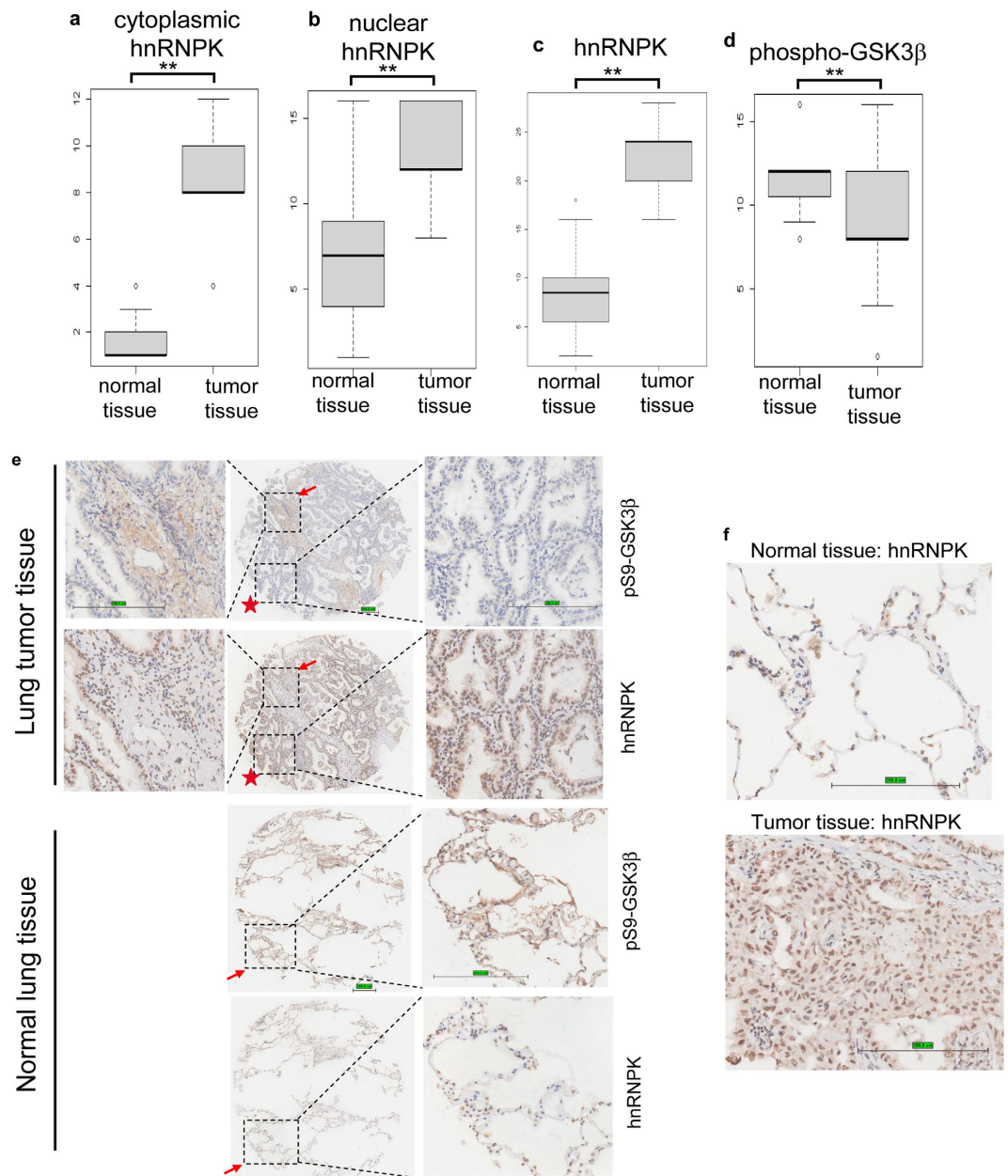


Figure 6. hnRNPk expression negatively correlates with Ser9 phosphorylated GSK3β in tissue microarrays (TMAs). TMAs of lung adenocarcinoma ($n = 52$) were immunohistochemically scored and statistically analyzed for the cytoplasmic hnRNPk (**a**), nuclear hnRNPk (**b**), total hnRNPk (**c**), and total phospho-GSK3β (**d**). $**p < 0.001$. (**e**) Immunohistochemical analysis of hnRNPk and phospho-GSK3β in representative normal lung tissue and lung tumor tissue on TMAs. The selected sections on the left and right panels were shown at $200\times$ magnification. The panels in the middle were shown at $50\times$ magnification. Brown color represents positive immune reaction. Scale bar: $200\ \mu\text{m}$. (**f**) Immunohistochemical analysis of hnRNPk in representative normal lung tissue and lung tumor tissue on TMAs. The selected portions were shown at $200\times$ magnification. Scale bar: $200\ \mu\text{m}$.

verify this hypothesis by trying to assess the effect of hnRNPk overexpression on PKC-GSK3β interaction using co-immunoprecipitation assays. However, it turned out to be impossible to detect PKC-GSK3β interaction by this method (data not shown). Consistently, whilst the Ser9 phosphorylation of GSK3β by PKC has been well documented in the literature^{33,47–49}, no report concerning the physical interaction between PKC and GSK3β could be found. This might probably due to the extremely dynamic interaction between these two proteins, as often observed between the kinases and their substrates. The verification of such hypothesis with more elaborated methodology would be of interest, and will be part of our future study.

Materials and Methods

Cell culture and transfection. Human non-small cell lung adenocarcinoma cell line H1299 (Cell Resource Center, Institute of life science Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Grand island, NY) supplemented with 10% fetal bovine serum (PAA Laboratories, Linz, Austria) at 37 °C in a humidified atmosphere containing 5% CO₂. H1299 cells at a 80–90% confluence were transfected with Lipofectamin 2000 (Invitrogen, Carlsbad, CA).

Expression and purification of GST fusion proteins. GST, GST-GSK3 β , GST-GSK3 β -S9A and GST-hnRNPK fusion proteins were produced and purified as described previously³⁴. The protein concentrations were estimated by a BCA assay (Beyotime Biotechnology, China).

Co-immunoprecipitation assay. H1299 cells transfected with Flag-hnRNPK or Flag-vector and treated with or without TRAIL (20 ng/ml, 8 hours) were lysed in lysis buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 5 mM NaF, 2 mM Na₃VO₄, 1 mM PMSF, 1% protease inhibitor cocktail (Roche)) for 30 min on ice. The co-immunoprecipitation assay were carried out with the protocols described previously⁵⁹ using the GSK3 β antibody (27C10, Cell Signaling Technology). The immune complexes were analyzed by Western blotting using antibodies against GSK3 β and Flag (A2220, Sigma).

Small interfering RNA assays. The sense strand sequences of siRNA used in this study are as follows: GSK3 β siRNA: 5'-AAGUAAUCCACCUCUGGCUACTT-3', hnRNPK siRNA1: 5'-UAUUAAGGCUCUCCGUACATT-3', hnRNPK siRNA2: 5'-CCUUAMGAUCCCAACUUUUTT-3' and control siRNA (NC): 5'-UUCUCCGAACGMGUCACGUTT-3'. These siRNAs were synthesized in GenePharma (Shanghai, China) and transfected into H1299 cells at 100 nM using Lipofectamin 2000 (Invitrogen). hnRNPK siRNA1 and hnRNPK siRNA2 were used in mixture. The interfered cells were harvested 48 hours after transfection.

GST pull-down assays. The GST pull-down assays were performed as described previously^{34,60,61}. Briefly, 1.5 mg whole cell lysate protein was incubated with 60 μ g GST-hnRNPK protein overnight at 4 °C on a rocker. Cellular proteins associated with GST-tagged fusion proteins were separated by SDS-PAGE, and analyzed by standard Western blotting using specific rabbit monoclonal GSK3 β antibody (27C10, Cell Signaling Technology, MA), phospho-GSK3 β (Ser9) antibody (9323, Cell Signaling Technology, MA). The amounts of GST-tagged fusion proteins were estimated by CBB (Coomassie Brilliant Blue) staining of SDS-PAGE gels.

MTT assays for cellular viability. Cellular viability was evaluated with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Beyotime Biotechnology, China) assay. H1299 cells (4000 cells per well) were seed into 96-well plate (TCP011096, JET, Guangzhou, China) and incubated for 16 h. Then, the cells were treated with TRAIL or/and LiCl (Sigma, 20 mM, 8 hours) as indicated. After treatments, the cells were incubated with MTT solution (final concentration 20 μ g/ml) for 4 h at 37 °C and the produced formazan crystals were dissolved in 150 μ l DMSO per well. Cellular viability was determined by measuring the absorbance of formazan crystals at 570 nm using a spectrophotometric microplate reader (ELX800, BioTek Instrument, USA). Three independent experiments were performed. The cellular viability of untreated controls was set as 100%.

Flow cytometry. H1299 cells after transfection of Flag-hnRNPK or knockdown of GSK3 β were treated with TRAIL (20 ng/ml, 8 hours) as indicated, then the cells were collected and stained with Alexa Fluor 488 Annexin-V/PI (HH-V13241, Invitrogen) following the manufacturer's protocol. Cells were subsequently sorted by a FACS Calibur flow cytometer (BD Biosciences) and analyzed by Flowjo software. Only living cells profiled using forward scatter (FSC) and side scatter (SSC) were counted. At least 10,000 cells within the gated region were counted and analyzed.

Detection of protein expression by Western blotting. Cells were transfected with Flag-GSK3 β , Flag-hnRNPK, siRNA target against GSK3 β or hnRNPK, or combined with treatments of TRAIL (Sino Biological Inc. Beijing, China), LiCl (Sigma), Gö6983 (1 μ M, Beyotime, China), Rottlerin (6 μ M, Millipore, USA), or CHX (10 μ g/ml, Amresco, USA) when it is indicated. Cells were then harvested and lysed with lysis buffer (20 mM Tris (pH7.5), 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β -glycerophosphate, EDTA, Na₃VO₄, leupeptin, and 1% protease inhibitor cocktail (Roche)). Whole cell lysates were separated by 10% or 12% SDS/PAGE.

Blots were probed with the specific antibodies against Flag (A2220, Sigma), GSK3 β (27C10, Cell Signaling Technology), phospho-GSK3 β (Ser9) (Cell Signaling Technology), hnRNPK (sc-28380, SANTA CRUZ), GAPDH (ZS-25778, ZSGB-BIO, China), β -catenin (Beyotime, China), Cleaved Caspase 3 (9664, Cell Signaling Technology), Cleaved Caspase 8 (9496, Cell Signaling Technology), c-FLIP (7F10, Alexis, Enzo life science, Switzerland). Horseradish peroxidase-conjugated secondary antibodies (ProteinTech Group) and enhanced chemiluminescence (ECL kit, Beyotime, China) were used to detect the expression of proteins.

Confocal microscopy assay. H1299 lung adenocarcinoma cells transfected with or without Flag-hnRNPK were treated with TRAIL (20 ng/ml). Immunofluorescence was performed as described previously^{34,60}. The primary antibodies (1:100 dilution) of hnRNPK, Flag and GSK3 β , and the Alexa Fluor 488 and Alexa Fluor 594 conjugated secondary antibodies (ZSGB-BIO, China) were used for observation. DAPI staining was used to determine the morphology of cell nuclei.

The imaging experiments were carried out on laser scanning confocal microscopes (LSM700, Zeiss, Jena, Germany) equipped with a Zeiss Plan-Neofluar 40 \times /1.3 NA Oil Dic objective as described previously^{34,60}.

Tissue microarrays and Statistics analysis. The expression of hnRNPK and the Ser9 phosphorylation of GSK3 β was analyzed using two consecutively numbered commercial tissue microarrays (TMAs) of lung adenocarcinoma (catalog no. OD-CT-RsLug04-003, Shanghai Outdo Biotech, Shanghai, China), consisting of 53 paired lung adenocarcinoma and adjacent normal samples, 2 unpaired lung adenocarcinoma tissues. The TMAs came from 30 male and 25 female patients, and were mainly grade II in the pathological grading. During the experimental process, one sample of lung adenocarcinoma tissue was destroyed, the final 52 paired samples of adenocarcinoma and normal tissues were analyzed.

The immunohistochemical staining of TMAs were performed in Shanghai Outdo Biotech. Briefly, tissue sections (4 μ m) were incubated in a 63 °C oven for one hour. After deparaffinization and rehydration, antigen retrieval was carried out by boiling sections in citrate buffer (10 mM, pH 6.0) for 5 min. Then, endogenous peroxidase activity was blocked with 3% H₂O₂ for 15 min at room temperature. The slides were blocked with 3% BSA in PBS for 30 min and subsequently cultured with primary antibodies against phospho-GSK3 β (Ser9) (1:100 dilution) and hnRNPK (1:500 dilution), respectively. The immune complex was detected using a Dako EnVision™ Detection system (Dako Japan Ltd.). Nuclei were counterstained with hematoxylin. Images of TMAs were captured by Aperio Scanscope XT (Leica, Germany).

The results were evaluated independently by 2 pathologists according to both the intensity of protein staining and the percentage of positive cells. The intensity of protein staining was scored as 1 (negative), 2 (weak), 3 (moderate) or 4 (strong). Evaluation of the percentage of positive cells was scored as: 1 (0%), 2 (\leq 30%), 3 (31–60%), and 4 (>60%). The total score of each case was consisting of the score of the intensity of protein staining and the score of percentage of positive cells. The paired Student's t-test was employed to analyze the significance of phospho-GSK3 β or hnRNPK with tumor or pericancerous normal tissue. Correlation between the expression of hnRNPK and phospho-GSK3 β (Ser9) were estimated using the Spearman's rank correlation analysis.

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Author Contributions

X.G. and L.L. conceived and designed the experiments; J.F., Y.H., F.X., X.F., W.H., H.X. and X.L. performed the experiments; X.G., Q.L. and W.L. performed the scoring and statistics analysis for tissue microarray experiments; X.G., X.S., Q.-Y.H., Q.Z. and L.L. analyzed the data; X.G. and L.L. wrote the paper. All authors discussed and commented on the manuscript.

Additional Information

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