# 論文の内容の要旨

# Thesis Summary

論文題目　**Characterization of AAG8 as an oncoprotein**

（がん蛋白質AAG8の機能解析）

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**Chapter 1 General Introduction**

Dysregulation of signaling pathways by changes of gene expression contributes to hallmarks of cancer. AAG8 (aging-associated gene 8 protein, encoded by the *SIGMAR1* gene) was originally considered an enigmatic polypeptide and has recently been identified as a unique ligand-regulated chaperone protein. It is often found to be overexpressed in various cancers such as melanoma, colon cancer and gastric cancer. The chaperone domain of AAG8 is C-terminal to two putative transmembrane domains (residues 11-29 and 80-100) but contains a predicted membrane associated region (residues ~176-204) containing two cholesterol recognition motifs. As a widely expressed interorganelle signaling modulator, AAG8 has been profoundly elaborated in neurology, and mutations of AAG8 cause neurodegenerative diseases. Combined ex vivo and in vitro studies demonstrated that the neuroadaptation is caused by a persistent protein-protein association between AAG8 and Kv1.2 channels, a phenomenon that is associated to a redistribution of both proteins from intracellular compartments to the plasma membrane. However, the rationale of AAG8 in carcinogenesis has rarely been noticed.

AAG8 is predominantly expressed at the mitochondria-associated endoplasmic reticulum (ER) membrane (MAM) and distributes dynamically. It modulates both MAM-specific and plasma membrane proteins and mitochondrial metabolism. AAG8 knockout mice were created in 2009 and with this knockout mouse model, the hallucinogen N,N-Dimethyltryptamine (DMT) has been identified as an endogenous agonist of AAG8. Strangely, the mice demonstrated no overt phenotype. Exogenously, a plethora of ligands of AAG8 have been synthesized, however, few have been tested for their anti-cancer property. BD1047 and its analogue BD1063 are the two specific AAG8 antagonists which have been widely used to elaborate the functions of AAG8, especially in neuroscience. Consistently the tiny changes of phenotype in AAG8 knockout mice, 100 μM BD1047 (the routinely used concentration in vitro, I also used this concentration in my studies) treatment of mouse organotypic hippocampal slice cultures (normal cells) for 24 h did not affect cell growth. The current study focuses on the following two research topics:

**Chapter 2 Modeling Tandem AAG8-MEK Inhibition in Melanoma Cells**

Melanoma is a lethal cancer notable for its aggressive, metastatic, and chemo-resistant propensity. The

known environmental and genetic risk factors include ultraviolet radiation exposure, pigmentation, and nevus phenotypes. Recent efforts employing whole-genome sequencing or chemical genetic screen methods have identified a panel of candidate molecules, including both recurrently mutated or wild-type proteins and RNAs, which contribute to melanomagenesis. Nevertheless, more than half of melanomas express the mutationally activated BRAF (V600E, the most prevalent genetic alteration) oncoprotein, which triggers the BRAF-MEK-ERK signaling pathway (MAPK pathway), a key regulator of proliferation and differentiation. Consequently, inhibitors targeting the clinically validated class of molecular components of MAPK cascade have been developed and shown to have notable clinical effects for melanoma chemotherapy. Drug resistance presents a challenge to the treatment of cancer patients, especially for melanomas, most of which are caused by the hyperactivation of MAPK signaling pathway. Innate or acquired drug-resistant relapse calls for the investigation of the resistant mechanisms and new anti-cancer drugs to provide implications for the ultimate goal of curative therapy.

A systematic study revealed AAG8 mRNA overexpression up to above eightfold in melanoma versus normal skin, indicating its vital roles in melanomagenesis. I therefore wondered whether perturbing AAG8 functions could affect melanoma cell growth by investigating AAG8 antagonism in B16F1 (B16) cells, derived from mouse melanoma. B16 cells express high level of AAG8 exclusively in the cytosol. I found that specific antagonism of AAG8 efficiently suppressed melanoma cell growth and migration through the inactivation of the CRAF-MEK signaling pathway. In agreement with the recent notion that CRAF phosphorylation is dependent on MEK activity, it is thus theorized that AAG8 antagonism could block this positive feedback loop to restrict melanoma cell growth.

It was further demonstrated that melanoma cells could get resistance to AAG8 antagonist. An AAG8 antagonist BD1047-resistant B16 cell line (termed B16BR) was established and these cells expressed comparable AAG8 level with B16 cells. However, they exhibited altered morphology in both 2D and 3D cultures. I then explored the molecular mechanisms of the drug resistance and found that it was due to the refractory CRAF-MEK activity in these resistant cells. RAS activity was next examined to investigate whether the reactivation of CRAF-MEK signaling is caused by RAS reactivation. Surprisingly, although specific AAG8 antagonism dramatically reduced RAS-GTP level in B16 cells, RAS activation was largely abrogated in B16BR cells. These data strongly suggest that while RAS-CRAF-MEK signaling is efficiently suppressed by specific AAG8 antagonism in B16 cells, some other pathways, rather than RAS, have been triggered to substitute the function of RAS and maintain the refractory CRAF-MEK activity, which contributes to the drug resistance of B16BR cells. Although mutationally activated RAS is a common event in carcinogenesis, my findings suggest that RAS mutation might not be involved in AAG8 antagonists-induced drug resistance. These data also reveal the tricky mechanisms which cancer cells lacking oncogenetic RAS employ to generate drug resistance.

The above findings suggest MEK as a common culprit in maintaining melanoma survival in drug-existing microenvironment. MEK acts as a central mediator for anticancer effects and also for the resistance mechanism, leading to my proposal of tandem AAG8-MEK inhibition in melanoma cells. Combination of specific AAG8 antagonist and very low concentration of a MEK inhibitor cooperatively restricted the growth of drug-resistant cells. This drug combination has critical implications for reducing the drug side effects during clinical melanoma prevention. These data collectively pinpoint AAG8 as a potential target and delineate a promising drug combination strategy for melanoma therapy. Tandem

AAG8-MEK inhibition might be a powerful therapeutic approach for increasing the antitumor efficacy and decreasing the drug resistance of each single inhibitor.

**Chapter 3 AAG8 promotes carcinogenesis by activating STAT3**

To further confirm that AAG8 is indeed an oncoprotein, I then explored its intrinsic roles in cancer cells with the employment of gain- and loss-of-function approaches. I found that AAG8 promoted carcinogenesis both in vitro and in vivo. Specific AAG8 antagonism induced growth-suppressive phenotype of colorectal COLO205 cancer cells, pancreatic PANC1 cancer cells, and gastric AGS cancer cells in 3D Matrigel culture. In addition, AAG8 antagonism induced apoptosis of 3D-cultured COLO205 cells. Moreover, AAG8 overexpression promoted proliferation of both DLD-1 and AGS cells. In line with this, AAG8 knockdown with short hairpin RNA (shRNA) delayed DLD-1 cell proliferation and suppressed AGS cell growth in 3D culture, which also closely mimicked the phenotype changes observed with AAG8 antagonist. Interestingly, although AAG8 knockdown resulted in few to no alternations of the morphogenesis of colorectal HCT116 cancer cells in 3D culture, it increased their sensitivity to gemcitabine, a clinical cancer drug. In agreement with the data in vitro, AAG8 knockdown slowed xenograft tumor formation of DLD-1 cells in vivo. These results collectively illustrate the tumor-promoting roles of AAG8, imply that AAG8 serves as an oncoprotein, and strongly indicate AAG8 as a potential target for tumor chemotherapy.

For explaining the underlying molecular mechanisms of AAG8 in promoting carcinogenesis, I unanticipatedly discovered STAT3 inactivation in both PANC1 and AGS cells treated with BD1047. A time-dependent assay revealed that STAT3 activity began to decrease 3 hours after BD1047 treatment and was largely suppressed after 6 hours in mouse melanoma B16 cells. STAT3 is a transcription factor intensively been investigated in cancer, immunity and neurology. Upon phosphorylation, activated STAT3 translocates into the nucleus to initiate transcription. STAT3 hyperactivation is a feature of the majority of solid cancers. I supposed that AAG8 may act as a STAT3 activator to enhance cancer cell proliferation. Supporting this hypothesis, it was found that STAT3 Y705 phosphorylation level was increased by ectopic AAG8 expression in DLD-1 cells, while AAG8 knockdown decreased STAT3 activation in DLD-1 cells, as well as in AGS cells. These findings indicate AAG8 as an upstream STAT3 activator.

To identify whether AAG8 is a mediator of the well-established JAK-STAT3 signalling pathway, a SCADS (screening committee of anticancer drugs) screening was performed in DLD1 cells stably expressing both AAG8 and a luciferase STAT3 reporter, such that STAT3 activity could be monitored after drug treatment. Among totally 364 chemicals with 232 targets, an API4 (apoptosis inhibitor 4) inhibitor YM155, was found to dramatically (fold change $> 20$) decrease STAT3 activity in these cells. To further disentangle this event, DLD-1 cells with stable AAG8 knockdown were treated with or without YM155 for 12 h, followed by IL6 stimulation. As a result, IL6-induced robust STAT3 activation was largely abolished by YM155 treatment, confirming the STAT3 inhibitory effect of YM155 in SCADS screening and suggesting that YM155 disturbs the signalling activities, which are indispensable for IL6-induced STAT3 activation. In contrast, AAG8 knockdown contributes no change to IL6-induced STAT3 phosphorylation level, meaning that AAG8 activates STAT3 beyond IL6-dependency. Substantiating this conjecture, specific AAG8 antagonists BD1047 or its analog BD1063 failed to decrease IL6-induced

STAT3 activation in DLD-1 cells. To my surprise, AAG8 knockdown further diminished the remaining pSTAT3 in YM155-treated cells. Based on the above findings, I hypothesized that AAG8 knockdown could also decrease STAT3 activity in cells with inhibition of IL6/JAK pathway. As expected, similar results to YM155 treatment were obtained with two JAK inhibitors JAK inhibitor I and Ruxolitinib.

To analyze the temporal regulation of STAT3 by YM155 in AAG8-knockdown cells, we decreased the time of YM155 treatment to 6 h or increased it to 18 h. YM155 treatment for 6 h hardly affected API4; however, AAG8 knockdown led to decreased STAT3 activity in YM155-treated cells. In contrast, YM155 treatment for 18 h led to decreased API4 expression, dramatically lowered STAT3 activity, and even reduced total STAT3 level. Notably, AAG8 knockdown further abolished STAT3 activation and reduced API4 in YM155-treated cells. These data exclude the possibility that YM155 inhibits STAT3 activation dependent on AAG8-related signallings. These results together give rise to the conclusion that AAG8 alternatively activates STAT3 in addition to IL6/JAK pathway.

API4 is an anti-apoptotic protein, and its transcription can be concurrently modulated by several transcription factors such as STAT3, β-Catenin and YAP1. The stable API4 knockdown DLD-1 cell line could not be established, perhaps due to its cytotoxicity, as reported elsewhere. To investigate whether API4 is required for IL6-induced STAT3 activation, I transiently knocked down API4 in DLD-1 cells with two specific shRNAs. Surprisingly, API4 depletion was dispensable for IL6-induced STAT3 phosphorylation in these cells. Although YM155 has been principally regarded as an API4 inhibitor, its specificity remains uncertain. Since YM155 dramatically decreased IL6-induced STAT3 activation while API4 knockdown did not, I supposed that YM155 might employ other inhibition mechanisms for this inactivation. There are at least two pieces of evidence supporting this argument. Firstly, while YM155 treatment for 6 h did not decrease API4 expression, it had already cooperated with AAG8 knockdown to decrease STAT3 activation. Secondly, YM155 directly targets ILF3 (interleukin enhancer-binding factor 3), a transcription factor, to suppress API4 expression. Conclusively, some proteins besides API4 could be suppressed by ILF3-dependent YM155 treatment. In summary, YM155 appears to be a potent STAT3 inhibitor independent of its suppression on API4.

I then supposed that combining YM155 and AAG8 knockdown could more efficiently suppress STAT3 activation and cancer cell growth. Accordingly, YM155 treatment significantly limited DLD-1 cell growth in 3D culture, which was enhanced by AAG8 knockdown, suggesting the synergistic antitumor effects by combined inhibition of these two proteins. Similarly, AAG8 knockdown significantly slowed the proliferation of DLD-1 cells treated with the JAK inhibitor JSI-124 and JAK3 Inhibitor VI.

**Chapter 4 Conclusion**

This study characterizes AAG8, for the first time to our knowledge, as an oncoprotein in multiple types of cancers through investigating its cancer-promoting effects and the underlying mechanisms. I uniquely uncover the molecular clues that: 1. AAG8 antagonism exhibits anti-melanoma effects through, at least partly, the inhibition of the CRAF-MEK signaling activity; 2. AAG8 is an alternative upstream STAT3 activator in addition to IL6 pathway. Drug combination implications were obtained from these investigations and further proved to be efficient approaches for cancer therapy. This study provides the fundamental evidences for identification of AAG8 as a potential target for cancer prevention, and highlights the importance of ER proteins in contributing to carcinogenesis.