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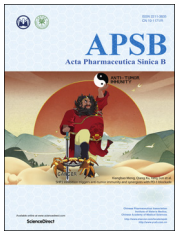

MEETING REPORT

Highlights of the 2nd International Symposium on Tribbles and Diseases: tribbles tremble in therapeutics for immunity, metabolism, fundamental cell biology and cancer

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Abstract The Tribbles (TRIB) family of pseudokinase proteins has been shown to play key roles in cell cycle, metabolic diseases, chronic inflammatory disease, and cancer development. A better understanding of the mechanisms of TRIB pseudokinases could provide new insights for disease development and help promote TRIB proteins as novel therapeutic targets for drug discovery. At the 2nd International Symposium on Tribbles and Diseases held on May 7–9, 2018 in Beijing, China, a group of leading Tribbles scientists reported their findings and ongoing studies about the effects of the different TRIB proteins in the areas of immunity, metabolism, fundamental cell biology and cancer. Here, we summarize important and insightful overviews from 4 keynote lectures, 13 plenary lectures and 8 short talks that took place during this meeting. These findings may offer new insights for the understanding of the roles of TRIB pseudokinases in the development of various diseases.

1. Introduction

The Tribbles (TRIB) pseudokinases control multiple aspects of eukaryotic cell biology and have evolved unique features distinguishing them from all other eukaryotic protein kinases[1]. Understanding the roles of Tribbles proteins, which include human TRIB1, TRIB2 and TRIB3, in disease initiation and development provides better opportunities for targeted-therapies in the future. Due to the absences of the conserved kinase catalytic domain or ATP binding sites, TRIBs are described as pseudokinases[2]. TRIBs, as key adaptors, play important roles to regulate several important signaling pathways and control the quality of many proteins[3]. Recent advancements focused on the roles of TRIBs in immunology, metabolism, fundamental cell biology and tumorigenesis[4–7]. Emerging evidences indicates that targeting TRIBs might also provide therapeutic opportunity for many different diseases[8,9].

The 2nd International Symposium on Tribbles and Diseases was held between May 7–9, 2018 in Beijing, China. This international meeting was sponsored by the Chinese Academy of Medical Sciences (CAMS) and State Key Laboratory of Bioactive Substance and Function of Natural Medicines (BSFNM). The organizers invited worldwide scientists who focused on the research of TRIBs protein in many different perspectives. The meeting was composed of three major sessions, including “Tribbles and Immunity & Metabolism”

chaired by Prof. Warren Pear and Dr. Zhuowei Hu, "Tribbles and Fundamental Cell Biology" chaired by Dr. Karen Keeshan and Prof. Takuro Nakamura, and "Tribbles and Cancer" chaired by Prof. Patrick Eyers and Prof. Leonard Dobens.

The meeting consisted of 4 keynote lectures, 13 plenary lectures and 8 short talks. This report summarizes the major highlights presented at the 2nd International Symposium on Tribbles and Diseases. With a focus on the most recent advances in the fields of immunity, metabolism, fundamental cell biology, and cancer, the programs covered a wide range of important topics, including atherosclerosis, obesity, fibrotic diseases, inflammation, normal blood cells differentiation, signaling in *Drosophila* and *Caenorhabditis elegans* development, metabolism and signaling in cancer, metastasis, and protein-quality control.

2. Opening keynote lecture: "The interface between signaling and metabolism in cancer" by Dr. Arkaitz Carracedo

Dr. Jiang, the Director of the Institute of Materia Medica (IMM, Beijing, China) at the Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College (PUMC), greeted all the attendees at the international Tribbles meeting, which was hosted by Dr. Zhuowei Hu's group from IMM. Dr. Endre Kiss-Toth, co-chairman of the organizing committee, welcomed the worldwide attendees, and then introduced Dr. Arkaitz Carracedo from Spain to present the first keynote lecture. Dr. Carracedo's laboratory[10–13] is focusing on investigating the contribution of cell metabolism to cancer cell biology, and the development of more selective and effective therapies for cancer. Dr. Carracedo presented his recent work on metabolic reprogramming in prostate cancer. Cellular transformation and cancer progression are accompanied by metabolic changes, and there are different strategies to study metabolism in the context of cancer. Firstly, bioinformatics analysis is a good strategy to uncover novel metabolic regulators. The function of the transcriptional co-activator PGC1A as a prostate tumor suppressor could be contextualized to promote the exploitation of publicly available transcriptomics datasets in the context of Tribbles family proteins. Secondly, genetic mouse model is a faithful model of the human disease. The data on the metabolomics analysis of murine models and human prostate cancer specimens led to the discovery of a novel molecular connection between the oncogenic mTORC1 pathway and polyamine biosynthesis[12]. This metabolic route could be used to exemplify the identification of cancer relevant therapeutic targets, as well as the complexity in the cross-talk between cell signaling and metabolism[11]. At last, Dr. Carracedo emphasized the potential of investigating the connection of oncogenic signals and metabolic networks for the establishment of better stratification strategies and therapeutic initiatives.

3. Session on "Tribbles and Immunity and Metabolism" chaired by Dr. Warren Pear and Dr. Zhuowei Hu

3.1. Key lecture on "angiopoietin-like 4 protein in cancer metastasis" by Dr. Naguan Soon Tan

Metastasis accounts for most cancer related deaths. However, this process remains one of the most enigmatic aspects of the disease[14]. The epithelial-mesenchymal transition (EMT) is associated with key transcriptional changes during the metastatic process. Dr. Naguan Soon Tan focused on the requirement of metabolic reprogramming for epithelial-mesenchymal transition. He first introduced the *in vitro* models of EMT from his recent study[15], which identified angiopoietin-like 4 (ANGPTL4) as a key player that coordinates an increase in cellular energy flux crucial for EMT *via* an ANGPTL4/14-3-3γ signaling axis (Fig. 1). ANGPTL4, a secreted glycoprotein, binds to specific integrins, cadherins and claudins, but not Tie receptors and VEGFR[16]. ANGPTL4 was found to protect against severe proinflammatory effects of saturated fats[17]. Elevated ANGPTL4 expression is found in many cancer subtypes, including breast cancer, lung cancer and gastric cancer[15,18]. Dr. Tan discussed how augmented energy charge status enhances metastasis. ANGPTL4 knockdown was found to suppress an adenylate energy charge elevation, delay EMT, and reduce cancer metastasis to the lung and liver. Unbiased kinase inhibitor screens and ingenuity pathway analysis revealed that ANGPTL4 regulates the expression of 14-3-3γ adaptor proteins *via* the phosphatidylinositol-3-kinase/AKT and mitogen-activated protein kinase signaling pathways, which culminate in the activation of transcription factors, CREB, cFOS and STAT3. Moreover, an increase in an 'adenylate energy charge' by ANGPTL4 protein enhances EMT by inducing 14-3-3γ expression. The ANGPTL4/14-3-3 signaling axis consolidated cellular bioenergetics and stabilized critical EMT proteins to coordinate energy demand and enhanced EMT competency and metastasis, through interaction with specific phosphorylation signals on target proteins. Dr. Tan ended his talk by sharing some thoughts about the new ANGPTL4/14-3-3 signaling axis during the EMT.

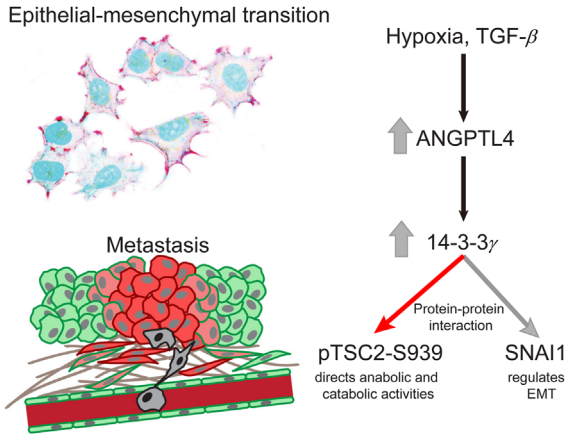

Figure 1 ANGPTL4:14-3-3 signaling axis integrates protein–protein interactome with kinome network to synchronize cancer cell metabolic activities in EMT. Epithelial-to-mesenchymal transition (EMT) plays crucial roles in the metastatic dissemination of carcinomas. Using *in vitro* and *in vivo* EMT models to compliment stage-specific human cancer biopsies, we have identified angiopoietin-like 4 (ANGPTL4) as a molecular driver of EMT-enriched metabolic changes for EMT competency. ANGPTL4 upregulates the expression of 14-3-3γ (herein called the ANGPTL4:14-3-3γ axis) that ensures metabolic flexibility to secure ample adenylate cellular energy to fuel various EMT-associated biological processes. The elevated 14-3-3γ adaptor protein coordinates cellular metabolic activity *via* interaction with tuberous sclerosis complex 2 (TCS2) and SNAI1. The 14-3-3γ stabilizes SNAI1-mediated repression of E-cadherin. TSC2 is an important gatekeeper of cellular metabolism that integrates extracellular signals to direct anabolic or catabolic activities. The 14-3-3γ:TSCS939 complex formation ensure the continued inhibition of TSC2S93.

3.2. *Plenary lecture on "TRIB1 mediated regulation of Treg function and kidney transplantation" by Yodit Feseha*

Regulatory $CD4^+$ T lymphocytes (Treg) are key players in the control of the immune system and thus play important roles in allograft survival in different experimental models and in human renal transplantation. In renal transplanted patients experiencing chronic antibody-mediated rejection (CAMR), it was discovered that a decreased blood Treg frequency associated with an incomplete forkhead box transcription 3 (*FOXP3*) promoter demethylation, the central transcription factor of Treg[19]. A meta-analysis where a combination of 6 genes were able to discriminates tolerant patients from stable patients[20,21]. The *TRIB1* gene was demonstrated to serve as a diagnostic biomarker for CAMR[22] and TRIB1 is over-expressed in Treg compared to counterpart naïve T cells and interacts in the nucleus with FOXP3[23]. TRIB1 may participate as part of a multiprotein regulatory complex driven by FOXP3, and modulates a downstream transcriptional program that is essential for Treg homeostasis, stability, proliferation and function. In order to address the role of TRIB1 in Treg, a mouse strain was developed with Tamoxifen conditional deletion of *Trib1* specifically in Treg. While *Trib1* over-expression impaired activated $CD4^+$ lymphocytes proliferation, their preliminary results highlighted *Trib1* deletion in transgenic mice was associated with expression alterations of several key genes.

3.3. *Plenary lecture on "Trib1 regulates eosinophil fate and identity by restraining the neutrophil program" by Dr. Warren Pear*

The evolutionarily conserved group of TRIB pseudokinases have been shown to regulate multiple cellular events including those involved in normal and malignant haematopoiesis[4]. Like their human counterparts, the three murine Tribbles gene homologues, *Trib1*, *Trib2* and *Trib3* are characterized by conserved motifs, including a pseudokinase domain and a C-terminal E3 ligase-binding domain. Dr. Warren Pear focused on the role of TRIB (murine Tribbles homologues) proteins in mammalian haematopoiesis and leukaemia. The TRIB proteins show divergent expression in haematopoietic cells, probably indicating cell-specific functions. The roles of the TRIB proteins in oncogenesis are also varied and appear to be tissue-specific. In their ongoing study, Dr. Pear utilized *Trib1* KO mice to identify the roles of TRIB1 in several cell types. In particular, the function of TRIB1 was investigated in mediating eosinophil lineage choice and differentiation from multipotent hematopoietic progenitors.

3.4. *Short talk on "Myeloid TRIB1 controls experimental atherosclerosis" by Dr. Jessica Johnston*

In Genome Wide Association Studies (GWAS), *Trib1* was identified as a potential regulator of plasma lipid levels and a risk factor for myocardial infarction (MI)[24–26]. Studies using *Trib1* full body- and liver-specific knockout mice had shown that hepatic expression of *Trib1* reduces circulating lipids[27]. Additionally, *Trib1* had been shown to be a regulator of alternatively activated macrophage polarization. However, no study directly evaluated the role of myeloid *Trib1* (m*Trib1*) in atherogenesis. Myeloid-*Trib1* deficient ($m*Trib1*^{KO}$) and overexpression ($m*Trib1*^{Tg}$) mouse strains were used to determine the role of m*Trib1* in atherosclerosis. To distinguish between metabolic and inflammatory drivers of atherosclerosis, bone marrow from these strains were transplanted into lethally irradiated $ApoE^{-/-}$ mice and fed on a Western diet for 12 weeks. Based on their initial results, Dr. Jessica Johnston suggested that *Trib1* is a potent regulator of atherosclerosis *via* regulating oxLDL uptake and subsequent foam cell formation in plaque macrophages.

3.5. *Plenary lecture on "Impact of Tribbles 1 and 3 on metabolism and inflammation" by Dr. Marcel Scheideler*

The *Tribbles* genes consists of three highly conserved mammalian homologues, each with its own distinctive functional properties in metabolism, inflammation and metabolic pathologies, including key roles in diabetes and obesity. TRIB3 levels are induced in liver under fasting conditions, as well as in livers of diabetic *db/db* mice. Hepatic overexpression of *TRIB3* in amounts comparable to those in *db/db* mice promoted hyperglycemia and glucose intolerance. TRIB3 has been identified as critical regulator of hepatic glucose homeostasis *via* negatively modulating AKT, thus contributing to insulin resistance in individuals with susceptibility to type 2 diabetes[28,29]. In white adipose tissue (WAT), TRIB1 was found to be significantly elevated in expression upon acute and chronic inflammation. In adipocytes, TRIB1 is under the control of NF-κB cytokine signaling, and TRIB1 controls pro-inflammatory gene expression in a cell-autonomous manner. Dr. Marcel Scheideler suggested that TRIB1 may be involved in the communication between metabolic (adipocytes) and immune cells in WAT, thereby contributing to WAT inflammation in response to activated WAT-associated immune reactions. As pro-inflammatory signaling is typically increased in sepsis, insulin resistance, and obesity-related type 2 diabetes, the cytokine-inducible TRIB1 function in WAT might provide a molecular rationale for the amplification of systemic inflammation. Moreover, TRIB1 haploinsufficiency protects against high-fat diet-induced obesity, indicating an impact on energy metabolism.

3.6. *Short talk on "TRIB3, a regulator of lipid handling and metabolic syndrome" by Zabran Ilyas*

Obesity is a major risk factor for many facets of metabolic diseases including diabetes, hypertension and cardiovascular disease[30]. TRIB3 has been implicated in these metabolic processes[30]. However, current insight into the role of TRIB3 in various organs remains limited. A systemic approach was used for the first time on a whole body $Trib3^{ko}$ mice to decipher a role for *Trib3* in metabolic dysfunction. A *Trib3* full body knockout ($Trib3^{ko}$) mouse was generated using a gene-trap system that disrupts the transcription of *Trib3* mRNA. Comparative gene expression microarray analysis was performed on the liver, adipose and muscle tissues from $Trib3^{ko}$ and wild-type littermates. Male $Trib3^{ko}$ mice were obese with elevated plasma levels of HDL and total cholesterol. These knockout mice also displayed a fatty liver phenotype, an increased macrophage influx and dysregulated proliferation in the adipose tissue. Microarray analysis revealed multiple metabolic pathways in the liver, adipose, and muscle tissues were altered, suggesting a dysregulated inter-organ communication underpinning obesity. Several key signaling regulators were identified alternation in the liver of $Trib3^{ko}$ compared with wild-type mice as evidenced by qPCR and Western blots. Mr.

Zabran Ilyas suggested that *Trib3* plays an important part in inter-organ communication that regulates lipid homeostasis.

3.7. Plenary lecture on "Tribbles-1, a GWAS locus for cardio-metabolic traits, exhibits adipose-specific regulation of plasma cholesterol, triglycerides, and adiponectin" by Dr. Robert Bauer

TRIB1, a novel mediator of plasma lipids and coronary artery disease in humans, was identified through genome-wide association studies[31,32]. Subsequent *in vivo* mouse work confirmed that hepatic Trib1 participates in the regulation of plasma lipids, yet little is known about metabolic roles for extra-hepatic Trib1[25,27]. SNPs near the *TRIB1* gene are significantly associated with circulating adiponectin levels in humans, suggesting a functional role for TRIB1 in adipose tissue. In light of this observation, the adipose-specific *Trib1* KO mice (*Trib1*_ASKO) were generated. It was found that *Trib1*_ASKO mice had altered plasma adiponectin levels. Interestingly, chow-fed *Trib1*_ASKO mice also exhibited altered total, HDL, and non-HDL cholesterol, as well as plasma triglycerides as compared to WT mice. Ongoing studies aimed to elucidate the mechanism through which adipose Trib1 protein regulates plasma cholesterol metabolism and adiponectin levels. Finally, an observed role for Trib1 in both adipose and hepatic regulation of plasma lipids suggested that common SNPs in the 8q24 region alter TRIB1 expression in both tissues. Using ENCODE data, they identified 10 candidate genomic regions and are currently testing them for transcriptional enhancer activity. Additionally, they are using pooled CRISPR library screens to understand tissue-specific regulation of *TRIB1* in adipocytes and hepatocytes. Dr. Robert Bauer presented the first *in vivo* validation of the human genetic association between TRIB1 and plasma adiponectin, and provided evidence suggesting that adipose TRIB1 contributed to the genetic associations observed in humans between *TRIB1* and multiple metabolic parameters.

3.8. Plenary lecture on "TRIB3 in erythroid and megakaryocytic differentiation" by Dr. Jorge Erusalimsky

The platelet-lowering agent anagrelide acts as a selective inhibitor of megakaryopoiesis[33,34]. Anagrelide targets a cellular event involved specifically in the megakaryocyte (MK) differentiation programme[33,35]. In a follow-up study, 328 annotated genes differentially regulated by anagrelide were identified. Many genes associate with platelet functions and the control of gene transcription. Prominent among the latter was *TRIB3*, whose expression increased in the presence of anagrelide[36]. Anagrelide induced the phosphorylation of eIF2, and increased the expression of the transcription factor ATF4, a known inducer of the *TRIB3* gene. Salubrinal, an effector of this pathway, also inhibited MK differentiation. These findings linked signaling through eIF2α/ATF4 to the anti-megakaryopoietic activity of anagrelide. Silencing *TRIB3* reduced the ability of anagrelide to inhibit MK growth. Furthermore, *TRIB3* was identified as a negative modulator of megakaryocytopoiesis in their recent study by using cellular model systems of haematopoietic lineage differentiation[37]. In primary cultures derived from human haematopoietic progenitor cells, thrombopoietin-induced megakaryocytic differentiation led to a time- and dose-dependent decrease in *TRIB3* mRNA levels. In the haematopoietic cell line UT7/mpl, silencing of *TRIB3* increased basal and thrombopoietin-stimulated megakaryocyte antigen expression, as well as basal levels of ERK1/2 phosphorylation. In primary haematopoietic cell cultures, silencing of *TRIB3* facilitated megakaryocyte differentiation. Based on the *in vitro* identification of *TRIB3* as a negative regulator of megakaryocytopoiesis, Dr. Jorge Erusalimsky suggested that this gene could be important for the regulation of platelet production *in vivo*[36,37]. At the end of his presentation, Dr. Erusalimsky also shared some thoughts about the role of *TRIB3* in erythroid and megakaryocytic differentiation.

3.9. Short talk on "Upregulation of Tribbles 1 in Oxidative Stress" by Dominika Grzesik

TRIB1 has been detected in atherosclerotic lesions. Human monocyte-derived macrophages (MDM) was found with the highest *TRIB1* RNA content contained, relative to those with the lowest *TRIB1* RNA content, an enrichment of genes involved in maintaining redox homeostasis. Conversely, mice with redox perturbation ($Nnt^{-/-}$) have increased adrenal *Trib1* RNA levels at 18 months of age, coincident with increased levels of reactive oxygen species. The relationship was discovered between *Nnt* deficiency and *Trib1* in adrenal cells and macrophages. These initial results provided the first evidence of cell-specific *Trib1* upregulation in response to chronic oxidative stress in adrenal cells and macrophages.

4. Session on "Tribbles and Fundamental Cell Biology" chaired by Dr. Karen Keeshan and Dr. Takuro Nakamura

4.1. Key lecture on "Substrate-induced ubiquitin ligase activation by the TRIB1 pseudokinase" by Dr. Peter Mace

Dr. Peter Mace's research is focused on understanding signaling networks that regulate how cells respond to stress. They are particularly interested in how phosphorylation and ubiquitination act as protein switches to control cell death and proliferation. TRIB1 can drive leukaemia development and is overexpressed across a range of other cancers[38]. The Tribbles family of pseudokinases recruit substrates—including metabolic proteins and transcription factors—to the COP1 ubiquitin ligase for ubiquitin-mediated protein degradation. CCAAT-enhancer binding protein (C/EBP) family transcription factors are a particularly relevant class of Tribbles-COP1 substrate in adipocyte and myeloid development, and in acute myeloid leukaemia. Several crystal structures of TRIB1 including an autoinhibited state and the TRIB1 pseudokinase in complex with the Tribbles recognition degron from C/EBPα were recently identified (Fig. 2). These structures showed that TRIB1 underwent a significant change relative to its substrate-free structure, with C/EBPα binding as a pseudo-substrate. Importantly, substrate-induced changes in TRIB1 engaged an allosteric network that linked substrate binding to release of the COP1 binding-motif of TRIB1 and formation of the COP1-TRIB1 ubiquitin E3 ligase complex. Such a model was consistent with biochemical experiments and molecular dynamics simulations. These findings offered a view of pseudokinase regulation with strong parallels to canonical kinase regulation and raised the possibility of small molecules targeting different conformations of Tribbles pseudokinases[39].

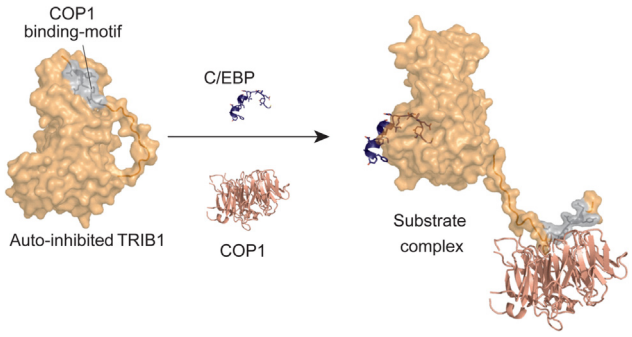

Figure 2 Schematic depiction of substrate-induced activation of TRIB1. In the substrate-free state, TRIB1 is able to sequester its own C-terminal COP1-binding motif. C/EBP binds as a pseudo-substrate and triggers conformational changes that release autoinhibition of TRIB1, favoring assembly of the TRIB1-COP1 complex.

4.2. Plenary lecture on "Repurposing covalent EGFR/HER2 inhibitors for on-target degradation of human Tribbles 2 (TRIB2) pseudokinase" by Dr. Patrick Eyers

Dr. Patrick Eyers first explained the difference between kinases and pseudokinases. A major challenge associated with biochemical and cellular analysis of pseudokinases is a lack of target-validated small molecules with which to probe their molecular function[2]. Human TRIB2 is a cancer-associated pseudokinase with a diverse interactome, which includes the canonical AKT signaling module, with which it interacts directly. There are also several lines of evidence that dysregulation of TRIB2 is relevant in solid tumors and myeloid- and lymphoid-derived human leukemias, making it a novel therapeutic target[1,40,41]. The non-canonical TRIB2 pseudokinase domain contains a unique cysteine rich region and interacts with a peptide motif in its own C-terminal tail, which was previously shown to drive interaction with cellular E3 ubiquitin ligases (Fig. 3). TRIB2 is a target for previously described small molecule protein kinase inhibitors, which were originally designed to inhibit the canonical catalytic domain of the tyrosine kinases EGFR/HER2[42]. Using a thermal-shift assay, TRIB2 ligands were discovered within the Published Kinase Inhibitor Set (PKIS), and then a drug repurposing approach was employed to classify ligands that either stabilize or destabilize TRIB2 *in vitro*. Remarkably, TRIB2 destabilizing agents, including the clinical covalent drug afatinib, led to rapid TRIB2 degradation in human cells, eliciting tractable effects on signaling and survival through direct binding to TRIB2[43]. These data revealed drug-leads for development of TRIB2-degrading ligands, which could also be invaluable for unravelling cellular mechanisms of TRIB2-based signaling. This study highlighted that small molecule-induced protein down-regulation through drug 'off-targets' might be relevant for other small molecules which serendipitously target Tribbles pseudokinases, including compounds repurposed from the clinic[44].

4.3. Plenary lecture on "Redundancy and specificity in Tribbles mediated signaling" by Dr. Endre Kiss-Toth

The *TRIB1* gene has been linked to several human pathologies, including lipid disorders and cardiovascular disease. It has also been established that *TRIB1* critically regulates M2-like macrophage differentiation and unpublished data have linked TRIB1 expression to increased atherosclerotic plaque formation[45]. Previous studies reported that TRIB1 protein is produced from a highly unstable mRNA, with a half-life shorter than 1 h, suggesting it may be subject to post-transcriptional regulation. Indeed, the *TRIB1* transcript includes a long, conserved 3UTR, enriched with miRNA-binding sites. The post-transcriptional regulation of *TRIB1* by miRNAs was investigated in the context of macrophage biology. Macrophages have been implicated in immune- and cardio-metabolic diseases; controlling their molecular responses through the activity of miRNAs and TRIB1 may represent an attractive therapeutic approach. Bimolecular fluorescence

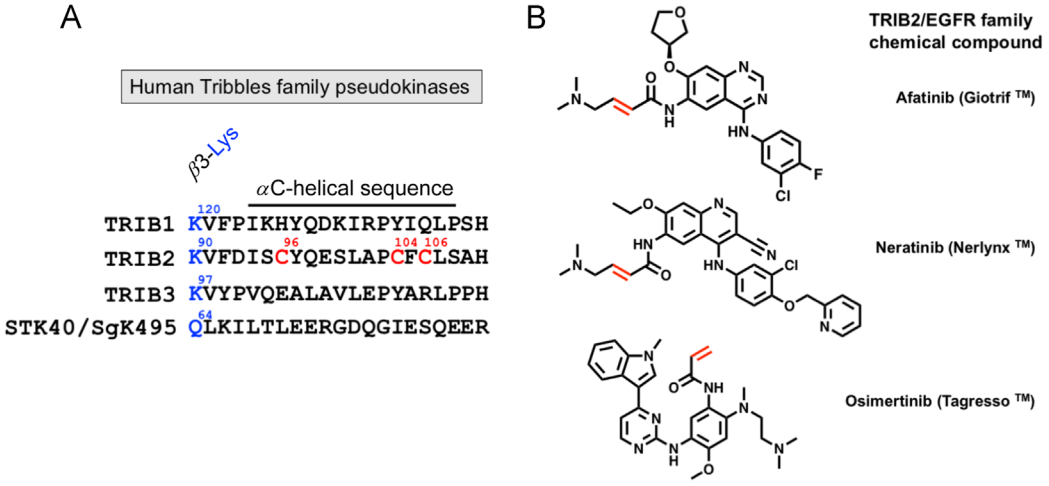

Figure 3 Human Tribbles family pseudokinases. (A) Alignment of the region encompassing the conserved-β3 Lys motif (which normally aids binding to ATP) and the atypical C-helix of human Tribbles (TRIB1-3) pseudokinases, and the less closely-related pseudokinase STK40/SgK495, which lacks a β3-Lys residue, and the N-lobe C-helix of which more closely resembles that of canonical protein kinases, such as PKA. The presence of three unique Cys residues (Cys96, Cys104 and Cys106, red) in human TRIB2 presents an opportunity for covalent conjugation to small molecules through a Thiol–Michael Addition reaction. (B) Clinically-approved irreversible covalent inhibitors of EGFR family kinases that are also shown to bind to human TRIB2. The double bond in each compound is shown in red for clarity.

complementation assays for Tribbles protein were also discussed. Tribbles proteins could bind to an overlapping set of MAPK-kinases (MAPKK) in live cells and dictated the localisation of the subsequent complexes. Binding studies in transfected cells revealed common regulatory mechanisms and suggested that Tribbles and MAPKs might interact with MAPKKs in a competitive manner. Computational modelling of the impact of Tribbles on MAPK activation suggested a high sensitivity of this system to changes in Tribbles levels, highlighting that these proteins were ideally placed to control the dynamics and balance of activation of concurrent signaling pathways[46].

4.4. Short talk on "Computational modelling of Tribbles complexes" by Juan Salamanca

Among the processes that occur in cells, direct protein–protein interactions (PPIs) are critical for many biological functions. Proteins do not act in isolation, and essentially all regulatory processes such as replication, transcription and signal transduction involved in biological responses are only made possible through multiple PPIs. It is therefore not surprising that dysregulation of PPIs is related to diseases such as cancer, neurodegenerative diseases, virus infection and metabolic disorders[47]. In this context, TRIB pseudokinases appear as an interesting family of proteins, involved in many PPIs. TRIB proteins had been proposed to play roles as "scaffolds" with other kinases (*e.g.*, TRIB3 interacts with MEK and AKT proteins) or as regulators of transcription factors and ubiquitylation (*e.g.*, TRIB C-terminal motif, COP1 and SIAH1) among others[1]. It is not surprising that dysregulation of TRIB proteins have been implicated in a variety of human cancers (AML, lung, hepatic and melanoma)[8]. Research into structure/sequence conservation and applying computational tools such as protein–protein docking together with experimental data will help to elucidate how these PPIs occur at an atomic level. Currently Salamanca's group is investigating the interactions between TRIB3 and some of its most interesting partners, the E3 ligase SIAH1 and the kinase PKB/AKT. Each of these studies aimed to solve different biological questions, the former related to the degradation of TRIB3 through an E3 ligase and the latter to understand TRIB3 as a potential regulator of the AKT signaling pathway. Salamanca proposed that identifying and understanding PPIs in more detail would give novel insight into biological processes and may lead to the development of new therapeutic approaches and drugs.

4.5. Short talk on "Disturbing TRIB3/MDM2 interaction attenuates pulmonary fibrosis by destabilizing transcription factor SLUG" by Dr. Xiaoxi Lv and Best Poster on "Disrupting the TRIB3/p62 interaction reduces liver fibrosis by restoring autophagic flux and suppressing exosome-mediated HSC activation" by Dr. Xiaowei Zhang

Pulmonary fibrosis (PF) is the pathological origin of incurable fibroproliferative lung diseases, but a few anti-PF therapeutics show certain promise. Recent studies have suggested that chronic or repeated lung injury causes failure of lung alveolar regeneration (LAR), which then interferes with the resolution of PF and can lead to anti-PF therapy resistance. Repetitive lung injury resulted in progressive accumulation of the stress-induced protein TRIB3, which caused LAR failure by interacting with E3 ligase MDM2 to suppress the ubiquitin-dependent degradation of snail family transcriptional repressor 2 (SNAL2) in alveolar epithelial cells. Disturbing the TRIB3/MDM2 interaction might restore LAR capacity and exhibits potent therapeutic efficacy against experimental PF. These findings suggested that TRIB3 play a key role on fibrotic diseases, which is consistent with Dr. Xiaowei Zhang's recent hepatic fibrosis study, and Dr. Zhang received the Best Poster Award at this meeting for presenting work on this topic. Recently, it was found that human cirrhosis tissue expressed higher levels of IL-17A, IL-17R and RORγt than control hepatic tissue. Targeting IL-17A with a neutralizing antibody attenuated cholestatic liver injury-induced hepatic fibrosis, restored hepatocyte autophagy activity, and increased animal survival. In addition, IL-17A also antagonism inhibited the phosphorylation of STAT3 in fibrotic tissues. After blocking STAT3 with the STAT3 inhibitor STATTIC or after *Stat3* siRNA, IL-17A failed to suppress the autophagy in AML-12 cells, indicating that IL-17A inhibited autophagy induction by phosphorylating STAT3[48]. The *Trib3* gene promoter could be activated by STAT3. Interestingly, the expression of TRIB3 was also much higher in human cirrhosis tissues compared with control tissues, which correlated positively with the expression of IL-17A. Moreover, immunostaining and Co-IP revealed an interaction between TRIB3 and p62/SQSTM1, an autophagic receptor, which could cause p62 accumulation and obstruct the autophagy-proteasome pathway. Accordingly, the anti-autophagy effect of IL-17A on hepatocytes was reversed by silencing *Trib3*, which were consistent with the *in vivo* findings that *Trib3* depletion protected against BDL-induced hepatic fibrosis, and restored hepatocyte autophagy activity. The IL-17A-STAT3-TRIB3 signaling pathway played a crucial role in the pathogenesis of hepatic fibrosis through regulating autophagy and blocking this pathway may provide therapeutic benefits for the treatment of hepatic fibrosis.

4.6. Plenary lecture on "Dissecting Tribbles signaling in C. elegans" by Dr. Yishi Jin

Caenorhabditis elegans (*C. elegans*) is an excellent model to investigate cellular stress responses including traumatic injury, neurotoxic stress, cytoskeletal stress and infection. Tribbles proteins are highly conserved eukaryotic pseudokinases that function to control kinase signaling and transcription in diverse biological processes, including worms, where NIPI-3 is the single Tribbles homolog. *C. elegans* Tribbles NIPI-3 was previously shown to activate host defense upon infection by promoting the conserved PMK-1/p38 mitogen-activated protein kinase (MAPK) signaling pathway. Despite the prominent role of Tribbles proteins in many species, our knowledge of their mechanism of action is fragmented, and the *in vivo* functional relevance of their interactions with other proteins remains largely unknown. The *NIPI*-3 null mutants characterizing studies showed that *NIPI-3* is essential for larval development and viability. Through analyses of genetic suppressors of *NIPI*-3 null mutant lethality, they found that NIPI-3 negatively controls PMK-1/p38 signaling *via* transcriptional repression of the C/EBP transcription factor CEBP-1. CEBP-1's transcriptional targets were identified by ChIP-seq analyses and found to be enriched in genes involved in development and stress responses[49]. Unlike its cell-autonomous role in innate immunity, NIPI-3 is required in multiple tissues to control organismal development. Dr. Yishi Jin concluded that NIPI-3 acts as a master regulator to inhibit CEBP-1 and the PMK-1/p38 MAPK pathway, keeping innate immunity in check and ensuring proper organismal development.

4.7. Plenary lecture on "Drosophila Tribbles identifies conserved motifs regulating subcellular trafficking" by Dr. Leonard Dobens

The fruit flies are an ideal model system for Tribbles research. Flies are useful to test conserved structure and various functions in

development and serve as a simplified system to screen for Tribbles pathway components. The single TRIB pseudokinase gene (*Trbl*) encodes a fly adaptor signaling protein that binds conserved targets including AKT kinase and CDC25 (cell division cycle 25) phosphatase to block cell growth and division in response to developmental and environmental signals[50,51]. Drosophila TRBL protein accumulated in the nucleus, cytoplasm and at the cell membrane, where it inhibited AKT phosphorylation to block insulin-mediated anabolism[50]. Misexpression of a membrane-tethered form of AKT (myr-AKT) resulted in strong mislocalization of TRBL to the inner leaf of the fat body cell membrane, demonstrating that AKT binded and recruited TRBL to the membrane *in vivo*. To identify domains in the TRBL protein that mediate its trafficking among cellular compartments, site-directed mutagenesis was used to identify a conserved motif ('ESLE286') that when mutated resulted in a TRBL protein that mislocalized strongly to the inner leaf of the cell membrane; this was abrogated by RNAi knockdown of *Akt*. A *TRBL* mutant (ESLE286G) was more stable than WT *TRBL*, an it more effectively recruited a tagged-WT *TRBL* to the membrane to strongly reduce cell growth and AKT phosphorylation-activation. An RNAi knockdown screen identified insulin pathway components upstream, downstream and in parallel to AKT that modulate the subcellular localization of *TRBL* (ESLE286G). To identify new *TRBL* targets, Dr. Leonard Dobens used a computer-based tool to measure the effect of *TRBL* misexpression interactors and identified two E3 ubiquitin ligases as candidate *TRBL* targets acting in a conserved Notch signaling pathway regulating cell size and tissue growth.

5. Session on "Tribbles and Cancer" chaired by Prof. Patrick Eyers and Prof. Leonard Dobens

5.1. Key lecture on "TRIB2 acts as a major driver of tumorigenesis and anti-cancer drug resistance" by Dr. Wolfgang Link

TRIB2 was identified as a novel oncogene in melanoma and as a driver of anti-cancer drug resistance. Intrinsic and acquired resistance to chemotherapy is the fundamental reason for treatment failure for many cancer patients. The identification of the dynamic molecular mechanisms involved in drug resistance or sensitization is therefore imperative. It was reported that TRIB2 ablates forkhead box O activation (FOXO) and disrupts the p53/MDM2 regulatory axis, conferring resistance to various chemotherapeutics and targeted drugs. TRIB2 suppression was exerted *via* direct interaction with AKT, a key signaling protein in cell proliferation, survival and metabolism pathways. Ectopic or intrinsic high expression of TRIB2 induced drug resistance by promoting phospho-AKT (at Ser473) *via* its COP1 domain. TRIB2 expression was significantly increased in tumour tissues from patients correlating with an increased phosphorylation of AKT, FOXO3a, MDM2 and an impaired therapeutic response. This culminated in an extremely poor clinical outcome. Dr. Wolfgang Link's study[52] revealed a novel regulatory mechanism underlying drug resistance and suggested that TRIB2 functions as a regulatory component of the PI3K network, activating AKT in cancer cells.

5.2. Plenary lecture on "Insights into TRIB1, TRIB2 and TRIB3 pseudokinases in haemopoiesis" by Dr. Karen Keeshan

All three Tribbles pseudokinases (*TRIB1, TRIB2 and TRIB3*) are important regulators of normal and malignant haemopoiesis[1,6]. Moreover, the relative abundance of each tribbles family member may be important for distinct oncogenic or tumour suppressor functions[1]. The expression profiles of *TRIB1, TRIB2 and TRIB3* were mapped in human and murine haemopoietic stem, progenitor and mature cells and in human leukaemia datasets (Fig. 4). *TRIB1-TRIB2* have an inverse expression relationship in normal haemopoiesis whereas *TRIB1-TRIB3* have a positive correlation. *TRIB3* expression is high in the dormant haemopoietic stem cell (HSC) population. These analyses supported a non-redundant role for each *Tribbles* member during normal haemopoietic differentiation. *TRIB1-TRIB2* display a significant negative correlation in myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML) subtypes, but not in acute lymphoid leukaemia (ALL). This inverse relationship is specific to certain subtypes of AML. A positive correlation exists in different leukaemia subtypes between *TRIB1-TRIB3*. Dr Karen Keeshan[6,53] concluded that there were significant differences in the range and variance of tribbles family members expression, and these insights may be important for the development of strategies to therapeutically target these genes in different types of leukaemia.

5.3. Short talk on "How does TRIB3 promotes acute promyelocytic leukemia and lymphoma progression?" by Dr. Ke Li

Acute promyelocytic leukemia (APL) is driven by the oncoprotein PML-RARα that antagonizes myeloid differentiation and promotes APL-initiating cell self-renewal. Either combing all-trans retinoic acid (ATRA) with arsenic trioxide (ATO), or chemotherapy, dramatically improves the prognosis of APL patients. Dr. Li reported that expression of TRIB3 pseudokinase associated positively with APL progression and therapeutic resistance. An elevated TRIB3 expression promoted APL by interacting with PML-RAR and suppressing its sumoylation, ubiquitylation and degradation. This repressed PML nuclear body assembly, p53-mediated senescence, cell differentiation, and supported cellular self-renewal. Genetically inhibiting Trib3 expression or combination of a peptide disturbing the TRIB3/PML-RAR interaction with ATRA/ATO eradicated APL by accelerating PML-RAR degradation. This study provided new insight into APL pathogenesis and suggests a new therapeutic option against APL[7].

5.4. Plenary lecture on "Role of TRIB3 in the regulation of cancer generation, progression and response to therapy" by Dr. Guillermo Velasco

TRIB3 regulates cancer generation, progression and response to therapy. Tribbles 3 is a member of the tribbles family of pseudokinases, which lacks conventional kinase activity. *TRIB3* participates in the regulation of multiple signaling pathways that are involved in different cellular processes like cell survival, proliferation or migration. Emerging data obtained during the last few years suggests that TRIB3 is a crucial modulator of tumorigenesis. Recent observations have shown that *TRIB3* plays a tumor suppressor role in several cellular and animal models of cancer through a mechanism that relies on the regulation of the mTORC2/AKT axis[54–56]. In addition, experiments performed with animal models showed that lesions in the prostate of *Pten*$^{+/-}$ mice were enhanced by *TRIB3* silencing[57]. Dr. Guillermo Velasco[58] then summarized their recent findings on the role of TRIB3 in the regulation of breast cancer, hepatocellular carcinoma and prostate cancer as well as on the role of this pseudokinase in the regulation of autophagy-associated cancer cell death in response to different stimuli.

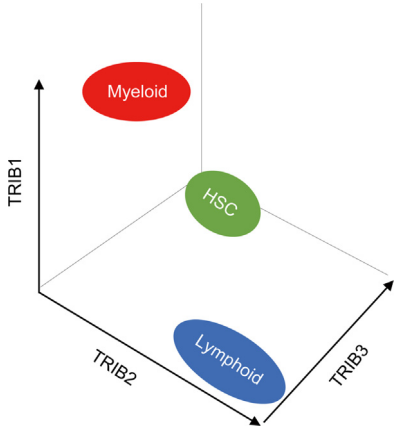

Figure 4 Schematic depicting the expression inverse and correlative relationship between TRIB1, TRIB2 and TRIB3 expression in haemopoietic cells[53].

5.5. Short talk on "TRIB3 promotes colorectal cancer stemness and progression by enhancing β-catenin/TCF4 transcriptional activity" by Dr. Shuang Shang

Aberrant activation of the *Wnt/β*-catenin pathway plays a fundamental role in colorectal cancer (CRC) pathogenesis. Elevated expression of TRIB3, a member of the tribbles family that is upregulated in response to many stressors, has been reported to be a negative prognostic marker of CRC, although the mechanism is elusive. It was investigated that whether elevated TRIB3 expression promotes CRC stemness and progression by crosstalk with WNT pathway. The initial results indicated that *TRIB3* promotes CRC tumorigenesis and progression in a PPI manner, which provided proof-of-concept for targeting this PPI as a therapeutic option against WNT-driven CRC[59].

5.6. Plenary lecture on "Transcriptional modulation by Trib1 in myeloid leukemogenesis" by Dr. Takuro Nakamura

Trib1 acts as a dominant oncogene for hematopoietic cells and also acts as a specific cooperative partner of *Hoxa9* in myeloid leukemogenesis[60,61]. *Hoxa9* also cooperates with Meis1, resulting in modification of target gene regulation and promotion of leukemic cell engraftment and expansion *in vivo*[62]. It is therefore of relevance to understand how the pseudokinase TRIB1 modulates HOXA9 activity during leukemia development. To clarify the TRIB1's function in Hoxa9-associated AML, HOXA9-induced leukemic cells derived from *Trib1* KO bone marrow with or without *Trib1* overexpression were generated. *Hoxa9*-binding loci are frequently associated with those of C/EBP in the absence of *Trib1* by ChIP-seq analysis. Given that TRIB1 induces degradation of C/EBP, it is predicted that TRIB1 may affect expression of HOXA9-regulated genes. Indeed, expression of 1164 genes were significantly upregulated in *Trib1*-overexpressing cells compared to *Trib1*-null cells ($FC > 2$, $P < 0.05$). Interestingly, ChIP-seq analysis for histone H3K27Ac revealed that distribution of super-enhancers was significantly modulated between two cell types. *Erg* and *Ptgds* are upregulated genes in which H3K27Ac signals are significantly increased by *Trib1* overexpression. Dr. Takuro Nakamura's results indicated that TRIB1 globally modulated the transcriptional program of HOXA9 and induced malignant phenotypes of AML.

5.7. Short talk on "New insights into clinical development of ABTL0812: a new antitumoral drug that induce autophagic cancer cell death by upregulating TRIB3 pseudokinase" by Dr. Jose Lizcano

ABTL0812 is a novel, first-in-class, small molecule that shows antiproliferative effects on tumor cells in phenotypic-based assays. Prof. Jose Lizcano[63] described the mechanism of action of this antitumor drug, which is currently in clinical development. ABTL0812 inhibited the AKT/mTORC1 axis, resulting in impaired cancer cell proliferation and autophagy-mediated cell death. *In silico* screening led Dr. Lizcano to identify the PPARs termed PPARα and PPARγ as the cellular targets of ABTL0812. He showed that ABTL0812 activated both PPAR receptors, resulting in upregulation of *TRIB3* gene expression. Upregulated *TRIB3* binded cellular AKT, preventing its activation by upstream kinases, resulting in AKT inhibition and suppression of the AKT/mTORC1 axis. Pharmacologic inhibition of PPARα/γ or *TRIB3* silencing prevented ABTL0812-induced cell death. ABTL0812 treatment induced AKT inhibition in cancer cells, tumor xenografts, and peripheral blood mononuclear cells in patients enrolled in phase I/Ib first-in-human clinical trial. ABTL0812 has a unique and novel mechanism of action, which defines a new and druggable cellular route that links PPARs to AKT/mTORC1 axis, where TRIB3 played a central role. Dr. Lizcano concluded that activation of this route (PPARα/γ-TRIB3-AKT-mTORC1) led to autophagy-mediated cancer cell death. Given the low toxicity and high tolerability of ABTL0812, their results supported further development of ABTL0812 as a promising anticancer therapy.

5.8. Plenary lecture on "Regulation of protein quality control as a strategy for research and development of anticancer drugs" by Dr. Zhuowei Hu

Cells detect and correct disordered proteomes through protein quality control (PQC) systems, which maintain protein homeostasis under stress[64]. Cellular senescence is often accompanied by a decrease in the ability of PQC. The accumulation of impaired and misfolded proteins can cause cell death and dysfunction. Autophagy and the proteasome system are the core machinery of PQC systems to regulate the degradation of different proteins and other biological macromolecules in cells. This system maintains protein homeostasis in cells, playing key roles in preventing oxidative stress and inflammation. Oncogenesis and multiple chronic inflammatory diseases involve the loss of balance in intrinsic growth and cellular senescence. Abnormal PQC contributes in an important way to the occurrence and development of cancer and chronic inflammatory diseases. Studies from Dr. Hu's team[65,66] have shown that TRIB3 could interact with autophagic cargo protein p62 to inhibit autophagic flux and promoted the development of solid tumors (Fig. 5). Furthermore, TRIB3 interacted with EGFR to promote its membrane recycling and inhibit its degradation during the malignant progression of lung cancer. Moreover, TRIB3 could also interact with proto-oncogene the PML-RARα product, to inhibit ubiquitin-proteasome activity and promote the development of promyelocytic leukemia[7]. Based on these findings, Dr. Hu's laboratory further demonstrated that the occurrence and development of tumors could be prevented and controlled through the regulation of the PQC function, offering a new potential approach for the development of innovative drugs.

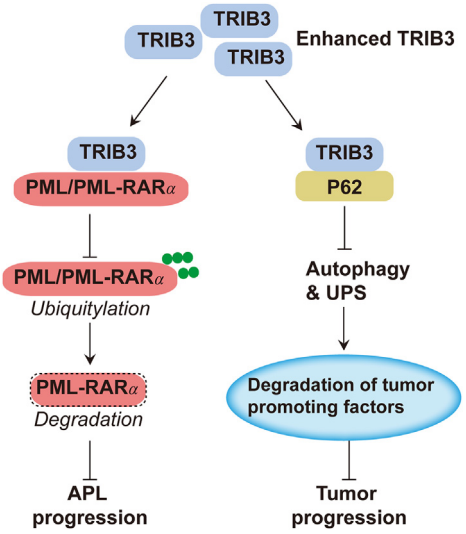

Figure 5 The role of TRIB3 in PML-RARα-driven leukemogenesis and tumor progression. Metabolic stresses increase TRIB3 expression to promote the interaction of TRIB3 and PML-RARα in APL cells, or to enhance the binding of TRIB3 and p62 in tumor cells. TRIB3 interacts with PML-RARα to interfere with PML-RARα ubiquitylation and degradation, which induces PML-RARα transcriptional activation and promotes APL. The TRIB3-p62 interaction impedes the binding of LC3 and ubiquitinated proteins to SQSTM1, which induces the autophagic flux inhibition, subsequently defective ubiquitin proteasome system (UPS). Dysfunction of two degradation systems result in a genome instability and a deposition of cancer-promoting factors. Thus, TRIB3 is critically involved in the pathogenesis of APL and tumor progression. Targeting TRIB3 is a potential therapeutic option for the treatment of cancer.

6. Summary

New findings reported at this meeting suggest that the TRIBs pseudokinase proteins regulate a number of key signal transduction systems and PPIs, many of which are known to be dysregulated in immune disease, metabolic disease or cancer[1]. Several of the talks presented at the 2nd International Symposium on Tribbles and Diseases reviewed recent important findings examining the roles of TRIB pseudokinases in different diseases. In the future, accurate dissection of the TRIB signaling machinery will provide a much better understanding the different molecular mechanisms controlled by TRIBs at specific stage of diseases, including clarification of the oncogenic and tumor suppressive functions of each of the individual TRIB proteins[1,3]. Cell permeable small molecules and PPI targeting peptides provide new therapeutic opportunities for many TRIB-associated diseases, including metabolic diseases and cancer[7,63,65]. The range of exceptional presentations, subsequent questions and insightful discussions will undoubtedly stimulate further studies on the regulatory mechanisms underlying TRIB-mediated signal crosstalk between metabolic tissues, immune cells and tumor cells, which will ultimately help advance the fields of chronic inflammatory diseases and cancer therapeutics.

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