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Acetyltransferase Tip60 inhibits the invasion and migration of renal cell carcinoma through JNK signaling

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Abstract: *Cancer is one of the major diseases affecting human health, and statistics show that about 90% of cancer patients die from tumor migration rather than primary tumor growth. The mechanism of tumor migration is still unclear, and most of the current studies are in vitro cell experiments, which can not fully reflect the real situation in vivo, so it is particularly important to use in vivo models for research. In vivo models of Drosophila melanogaster, we found that Tip60 inhibited cell migration induced by down-regulating the cell polar gene scrib and tumor invasion induced by $lgl^{-/-}/Ras^{V12}$. However, the deletion of acetyltransferase activity in Tip60 can induce cell migration through activation of JNK signaling pathway. We found that Tip60 inhibited JNK signals-mediated cell migration and tumor invasion through the acetylation of downstream transcription factor Fos, and inhibited the expression of downstream migration molecules such as MMP1. In this study, the role of Tip60-Fos in cell migration and tumor invasion was investigated, and the molecular mechanism of Tip60-Fos was elucidated, and corresponding validation was carried out in the samples of kidney cancer patients. These studies will further improve the regulatory network of tumor migration and provide new ideas for the treatment and drug target research of kidney cancer.*

Keywords: *Kidney Renal Clear Cell Carcinoma (KIRC), Drosophila melanogaster, JNK signal, Tip60, Fos*

1. Introduction

Tumor metastasis is one of the major challenges in cancer treatment and management[1]. Due to the distribution and complexity of metastatic tumors, it is often difficult to completely eradicate them. Treatment strategies usually include surgical resection, radiotherapy, chemotherapy, targeted therapy, immunotherapy and other comprehensive means, aiming at controlling tumor progression, alleviating symptoms, and prolonging patients' survival time[2, 3]. Tumor metastasis is a critical step in the development of cancer, which involves the spread and settlement of cancer cells from the site of the primary tumor to other parts of the body. The causes of death caused by tumor metastasis are related to tissue destruction and organ function impairment, vascular invasion and blood circulation diffusion, immune escape and immunosuppression, and multiple organ failure and other factors[4, 5]. In-depth study of the mechanism of tumor metastasis and the discovery of more biomarkers that mark tumor metastasis are the key to guide us to develop anti-cancer treatment strategies and prognostic evaluation, and to develop new anti-cancer drugs and therapies to improve the survival rate and quality of life of cancer patients by finding targets to interfere with the metastasis process.

Acetyltransferase Tip60 is a histone acetyltransferase that has attracted wide attention in the field of biology and oncology research[6]. Tip60 regulates gene expression and cell function by transferring acetyl groups to histones and other cytokines[7]. In the past few decades, Tip60 has made remarkable progress in the research of gene transcription, DNA repair and tumorigenesis[8-10]. In the study of cell biology,

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Tip60 has been found to be involved in the regulation of various cellular processes. It can regulate chromatin structure through acetylation modification and affect transcriptional activity of genes[11]. Tip60 also interacts with transcription factors, coactivators and chromatin remodeling factors to participate in the transcriptional regulatory network of genes[12, 13]. In addition, Tip60 is also involved in important physiological processes such as cell cycle regulation, DNA damage repair and apoptosis[14].

In oncology studies, the abnormal function of Tip60 is closely related to the occurrence and development of various tumors[15, 16]. On the one hand, Tip60, as a co-activator of transcription factors, plays an important role in key processes such as cell cycle regulation and DNA repair. Inactivation or mutation of Tip60 may lead to abnormal gene expression and reduced DNA damage repair ability, thus promoting the proliferation and mutation accumulation of tumor cells[17]. On the other hand, Tip60 is also involved in the activation of tumor suppressor genes and the regulation of tumor-related signaling pathways. Studies have found that Tip60 can interact with multiple tumor suppressor factors to promote their transcriptional activity and inhibit the proliferation and invasion ability of tumor cells[18]. In a variety of tumor types, the expression level of Tip60 is usually down-regulated, and the deletion or abnormal function of Tip60 is closely related to the clinical characteristics of tumor malignancy, prognosis and treatment response[19, 20]. Some mirnas and long-chain non-coding Rnas can affect biological behaviors such as proliferation, invasion and apoptosis of tumor cells by regulating the expression level of Tip60 or its interaction with other proteins, and these findings provide new clues for further understanding the mechanism of Tip60 in tumor occurrence and development[21]. Despite important advances in the study of Tip60 in biology and oncology, there are still some unanswered questions and challenges. First, the precise regulatory mechanisms of Tip60 in cellular processes are not fully understood, and further studies are needed to reveal its regulatory networks and signaling pathways. Second, the function of Tip60 may vary across tumor types and individuals, so more clinical samples and in vivo experiments are needed to verify its specific role in tumors.

Kidney Renal Clear Cell Carcinoma (KIRC) is one of the most common renal malignancies and accounts for the majority of adult renal tumors[22]. Over the past few decades, important advances have been made in the investigation of the pathogenesis and metastasis of renal clear cell carcinoma. The metastasis pathways of renal clear cell carcinoma mainly include local invasion and hematogenous metastasis. In terms of local invasion, the cells of renal clear cell carcinoma are highly invasive and have the ability to migrate, and can invade the surrounding tissues and spread to the renal pelvis and other sites[23]. At the same time, renal clear cell carcinoma can also spread to distant organs and tissues through hematogenous metastasis. Hematologic metastasis of renal clear cell carcinoma mainly carries cancer cells to distant places through blood circulation, and common metastatic sites include lung, bone, liver and lymph nodes[24]. Some studies have shown that the metastasis of renal clear cell carcinoma is closely related to the Epithelial-to-Mesenchymal Transition (EMT) process[25].

Multiple signaling pathways, such as JNK, Wnt/β-catenin, PI3K/AKT and TGF-β, are involved in EMT of renal hyaluronic cell cancer cells and play an important role in the metastasis of renal hyaluronic cell carcinoma[26-28]. Studies have found that the activation of JNK can promote the EMT process of renal hyaluronic cell cancer cells, thereby enhancing the migration and invasion ability of the cells[29]. JNK signaling pathway influences the interstitial structure and cell-cell interaction by regulating the expression of transcription factors and extracellular matrix degrading enzymes, thereby promoting the metastasis ability of renal hyaluronic cells. Secondly, JNK signaling pathway is also involved in angiogenesis and the formation of tumor microenvironment in renal hyaluronic cell carcinoma. Studies have found that the activation of JNK signaling pathway can promote the production of vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) by renal hyaluronic cell cancer cells, thus promoting tumor angiogenesis and remodeling of tumor microenvironment[30, 31], and these changes are conducive to the nutrient supply and metastasis of renal hyaluronic cell cancer cells. In addition, the activation of JNK can lead to the inhibition of apoptosis pathway, making renal hyaluronic cell cancer cells resistant to radiotherapy and chemotherapy[32, 33]. Inhibition of JNK signaling pathway has become a research focus in the treatment of renal hyaluronic cell carcinoma[34]. Some studies have found that inhibition of JNK signaling pathway can reduce the migration and invasion ability of renal hyaluronic cell cancer cells, inhibit angiogenesis and the formation of tumor microenvironment[29]. However, the specific regulatory mechanism of JNK signaling pathway and its role in renal hyaluronic cell carcinoma metastasis still need to be further studied and verified.

As a classic model animal, *Drosophila melanogaster* is of great significance and superiority in medical research[35]. Fruit flies have a number of characteristics and biological advantages that make them powerful tools for studying human diseases and related physiological processes: First, fruit flies have a short lifespan, a large amount of fecundity, and a rich resource of genetic variation, which makes

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them ideal model organisms in genetic research. Through genetic crossover and gene mutation techniques, researchers can easily analyze and manipulate the *Drosophila* genome to study gene function and regulatory mechanisms associated with human disease[36]. Second, the *Drosophila* genome has been fully sequenced and has a relatively small genome and simple genome structure. This makes the fruit fly an ideal model for studying genome structure, function, and regulation. Through genomics approaches, researchers can identify genes, regulatory elements, and signaling pathways associated with human disease and reveal their role in disease occurrence and progression[37]. Third, the fruit fly model also plays an important role in drug development. *Drosophila* has certain similarities in drug absorption, metabolism, and toxicity, and can therefore be used to evaluate the efficacy and safety of potential drugs. In addition, the *Drosophila* model can be used to screen drug libraries to find compounds with therapeutic potential for specific diseases[38]. Despite differences in appearance and physiological structure between fruit flies and humans, the basic biological and pathological mechanisms of many diseases are preserved in fruit flies[39]. By simulating a fruit fly model of human disease, researchers can delve into disease pathogenesis, causes and treatment strategies. For example, fruit fly models have been successfully used to study tumors, neurodegenerative diseases, cardiovascular diseases, and metabolic diseases[40]. In short, the significance and superiority of *Drosophila* as an in vivo model in scientific research cannot be ignored. It provides a cost-effective, operable and highly conservative research method for genetic inheritance and biological mechanisms, and provides important insights and research tools for us to deeply understand the occurrence of biological phenomena and diseases.

This study focused on the regulation mechanism of Tip60, an important acetyltransferase found in genetic screening, on cell migration and tumor invasion. Using a genetic screening model in *Drosophila*, we found that Tip60 inhibited cell migration and tumor invasion. Down-regulating the expression level of Tip60 or mutating its acetyl transfer function in *Drosophila* models can strongly activate JNK signaling pathway, trigger cell migration, and interact with the proto-oncogene Ras^{V12} to promote tumor invasion. Genetic interaction analysis showed that Tip60 acted on the downstream of JNK signaling pathway. Biochemical experiments confirmed that Tip60 could bind and acetylate the transcription factor Fos, thus inhibiting the activation of JNK signaling pathway. In addition, the expression of human Tip60 in *Drosophila* also inhibited the JNK signaling pathway and compensated for cell migration caused by the loss of Tip60 function in *Drosophila*, suggesting that the regulation of JNK signaling by Tip60 is evolutionarily conserved. Based on the above results, we believe that in the JNK signaling pathway, Tip60 acetylates Fos, thereby inhibiting the expression of downstream target genes (such as *mmp1*), and thus reducing the cell migration mediated by JNK signaling pathway. We also verified that the regulation of Tip60 on JNK signaling pathway is mechanically conservative in renal clear cell cancer cell lines and renal cancer tissue samples. Through these studies, we hope to further improve the regulatory network of JNK signaling pathway, reveal the role of JNK signaling in the occurrence and development of kidney cancer, and provide new ideas for the prevention of kidney cancer and the design of targeted drugs.

2. Methods

2.1 Drosophila genetics and stocks

Fly stocks were raised on standard cornmeal and agar medium at 25°C. For cell migration assay, larvae were reared at 29°C unless indicated. Fly strains used in this study are as follow: w^{1118}, *ptc*-Gal4, *UAS*-GFP, *UAS-scrib RNAi*, $UAS\text{-}Bsk^{DN}$, *UAS*-LacZ, *TRE*-RFP, fos^{1}, *UAS-bsk RNAi, UAS-dTAK1 RNAi, UAS-hep RNAi, UAS-foxo RNAi, UAS-fos RNAi*, lgl^{-}, Ras^{V12}, bsk^{1}, *UAS*-Bsk, *UAS*-Fos, *pnr*-Gal4 have been described previously[41-44]. *UAS*-Tip60^{E431Q}, *UAS*-Tip60^{WT}, h*UAS*-Tip60^{WT} were gifts from Dr. Lei Xue (Tongji University, China).

2.2 Immunohistochemistry

Antibody staining was performed according to standard procedures[45]. The following primary antibodies were used: mouse anti-MMP1 (3A6B4, 1:200, Developmental Studies Hybridoma Bank, DSHB), Rat anti-E-cad (DCAD2-c, 1:100, Developmental Studies Hybridoma Bank, DSHB) and mouse anti β-gal (40-1a, 1:500, Developmental Studies Hybridoma Bank, DSHB). The following secondary antibodies were used: anti-mouse CY3 (A11032, 1:1000, Cell Signaling Technology) and antiRat CY3 (104,086, 1:500, Jackson Immuno research).

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2.3 X-gal staining

Wing discs were dissected from third instar larvae in PBST (1× PBS pH 7.0, 0.1% Triton X-100) and stained for β-galactosidase activity as described[46].

2.4 RT-qPCR

Total RNAs were extracted from fifteen third instar larval tissues of indicated genotypes with Trizol (Ambion, Life Technologies, Carlsbad, CA, USA) following the protocol of RNA preparation kit, and quantitative polymerase chain reaction (qPCR) was performed using SYBR Green PCR Premix Kit (TaKaRa). The primers used are as follow (table 1) :

Table 1: Primer sequence used in this study.

Gene	Forward primer	Reverse primer
GAPDH	CTTCGCTCTCTGCTCCTCCT GTTCG	ACCAGGCGCCCAATACGACC AAAT
MMP1	TCCCCATGAACGAGGAA TTCC	AACCATCCAATCGGTAG TAGC
MMP2	GCAGTTAGAATAGGGG AGCTT	GGTCCAGTTTTTTTTTTTT TTTAAGTTAAG
MMP9	GCAGTTTAGAATTCCT ACGCT	GGTCCAGTTTTTTTTTT TTTTTGGT
MMP10	CGCAGTAATACTGTC AGGT	TCATGCTTAGTCCAC TGTCTGT

2.5 Cell culture and Transfection

S2 cells were cultured in *Drosophila* Schneider's Medium (Gibco, 21720, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen) at 25°C in a humidified air atmosphere. Transient transfection was performed with the Effectene Transfection Reagent (Qiagen, 301427, Germany) according to the manufacturer's instruction. The *actin*-Gal4 plasmid was co-transfected with the pUAST constructs for all the transfections in S2 cells. Human 293T cells and renal clear cell adenocarcinoma cell line 786-O were maintained at 37°C in DMEM (Gibco) containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified incubator with 5% CO_2. Cells were transiently transfected using Lipofectamine 2000 (Invitrogen, 11668019, USA) following the instruction. Lentiviral production, purification, titration, and infection of overexpressing constructs were conducted as described[47, 48].

2.6 Immunoblotting

Cells were harvested and washed in ice-cold PBS, then lysed with RIPA lysis bufer (WELLBIO, WB0101, China) supplemented with protease inhibitor cocktails (Yeasen, 20124ES03, China) on ice for 30 min. Cell lysates were then centrifuged at 15,000 rpm for 10 min at 4℃. Proteins were separated by SDS-PAGE following standard procedures. The primary antibodies were used as follows: Rabbit anti-JNK (CST, 5258T, 1:2000), Rabbit anti-SAPK/JNK (CST, 9252, 1:2000) and Rabbit anti-phospho SAPK/JNK (CST, 9251, 1:2000).

2.7 Wound Healing Assay

The wound healing assay was performed as previously described[49]. After 48 h of siTip60 plasmid transfection, 786-O cells were seeded on 6-well plates and reached 100% confluence. The cells were starved overnight, and a wound was made by using a sterile 200µL pipette tip to scratch the artificial wounds. The cells were washed with PBS 3 times. Wound healing was observed by microscopy after 24, 48, and 72 h.

2.8 Immunohistochemical (IHC) analysis

The xenograft tumor samples were embedded in paraffin, cut into 4-µm sections in a microtome, and incubated with an antibody against Tip60 (Proteintech, Wuhan, China) with DAB staining according to

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the manufacturer. The percentage of apoptotic cells was recorded using an Olympus DP73 digital microscope camera (Olympus, Tokyo, Japan).

2.9 UALCAN and GEPIA cancer databases

The UALCAN cancer database (http://ualcan.path.uab.edu/analysis.html) is a comprehensive web source that provide the data from The Cancer Genome Atlas (TCGA). The GEPIA database (http://gepia2.cancer-pku.cn/#index) contains data from TCGA and Genotype-Tissue Expression (GTEx) project.

2.10 Statistical analysis

Statistical analysis was performed with GraphPad Prism 8 software. The data were analyzed by two-tailed unpaired t-test or one-way ANOVA with corrected Bonferroni's Multiple-Comparison Test to calculate statistical significance in all experiments (**P* < 0.05; ***P* < 0.01; ****P* < 0.001, *****P* < 0.0001; ns, no significant difference). The experiments were repeated at least three times.

3. Results

3.1 Genetic screening showed that Tip60 overexpression inhibited cell migration

In recent years, researchers have found that the wing disc can serve as a powerful model for studying cell migration in the body. The disc is simple in structure, composed of only one layer of epithelial cells, and the cells are closely arranged and the polarity distribution is orderly. *Patched*-gal4 (*ptc*-Gal4) specifically down-regulates the expression of polar genes (such as *scrib, dlg, lgl*) along the antero-rear axis of adult wing discs, resulting in cell migration and invasion phenotypes. The emergence of this migration model has further accelerated the discovery of cancer-related migration genes in fruit flies, and geneticists can easily use RNAi or overexpressed fruit fly strains to study the relationship between target genes and cell migration and cancer development (Figure 1). It is worth mentioning that these experimental results have also been verified in mammals, affirming the reliability and effectiveness of using fruit flies to study cancer occurrence.

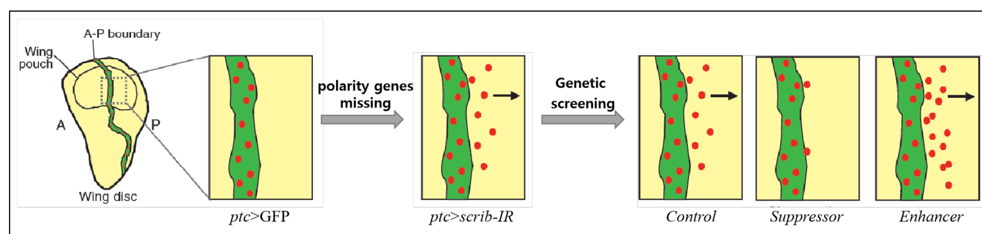

Figure 1: Screening model of cell migration genes in Drosophila melanogaster.

When screening by cell migration phenotype induced by *ptc>scrib-IR* (Figures 2C and O), we found that overexpression of Tip60 significantly inhibited cell migration (Figures 2E and Q) compared with LacZ control (Figures 2D and P). Overexpression of dominant-negative form of Bsk (Bsk^{DN}) as a positive control also well inhibited cell migration (Figures 2F and R). RNAi of *Tip60*, on the other hand, significantly promoted down-regulation of *Scrib*-induced cell migration phenotypes (Figures 2G and S). An important initial step in cell migration is the degradation of the Basement membrane (BM), which destroys the tissue integrity. Metalloproteinase 1 (MMP1) is an important protein in the degradation of basement membrane, and the expression of MMP1 is significantly increased in the initial stage of cell migration. Therefore, the expression of MMP1 can be used as an important molecular marker of cell migration. While inducing cell migration, *ptc>scrib-IR* activated a large amount of MMP1 expression (Figures 2I and O). Compared with LacZ control (Figures 2J and P), this activation could be significantly inhibited by overexpression of Tip60 (Figures 2K and Q), while down-regulated *Tip60* was enhanced (Figures 2M and S). Therefore, Tip60 negatively regulates cell migration and MMP1 activation caused by polar gene inactivation.

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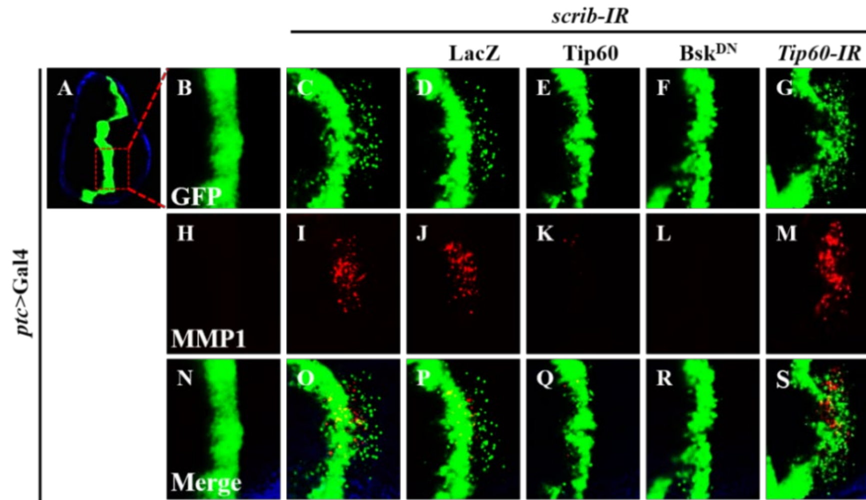

Figure 2: Tip60 inhibits cell migration caused by deletion of cell polarity genes.

3.2 Loss of Tip60 acetyltransferase activity leads to cell migration

We then investigated whether down-regulation of Tip60 was sufficient to induce cell migration. Considering that Tip60 is an important histone acetyltransferase, we speculated that the acetyl transfer function of Tip60 may be involved in cell migration. Therefore, *ptc*-Gal4 was used to express Tip60 (Tip60^{E431Q}, a dominant negative HAT-defective version of Tip60) with point mutations in the acetyl transfer domain. It was found to cause A large number of cells to migrate to the rear at the A/P junction of the wing disc in adult worms, and MMP1 was also highly activated (Figures 3A, D, and G). Epithelial to Mesenchymal Transition (EMT) process can cause remodeling of Actin and decrease of E-cadherin (E-cad). We have tested the expression of both. It was found that overexpression of Tip60^{E431Q} significantly induced Actin remodeling (Figures 3B, E, and H), and the expression of E-cad was also significantly decreased (Figures 3C, F, and I). Therefore, the loss of Tip60 acetyltransferase activity can fully induce the occurrence of cell migration.

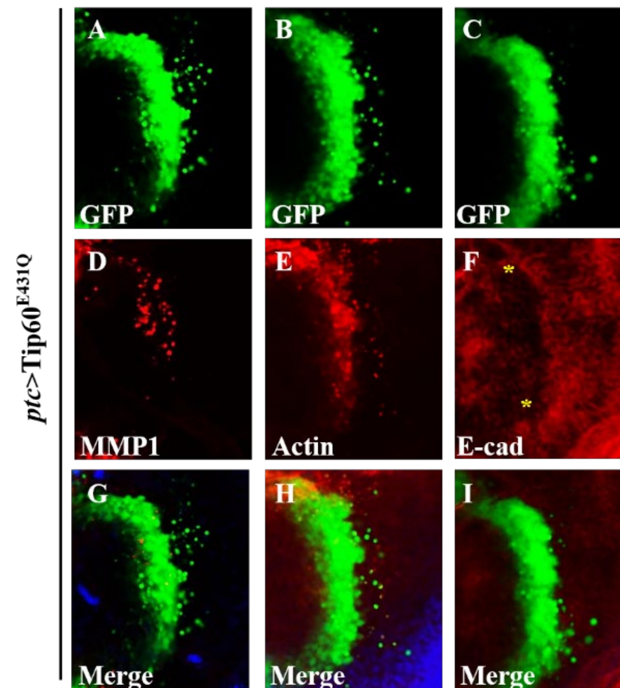

Figure 3: Loss of Tip60 acetyltransferase activity leads to cell migration.

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3.3 The cell migration caused by the loss of Tip60 acetyltransferase activity depends on the JNK-Fos signaling pathway

JNK signaling pathway is closely related to cell migration and tumor invasion, and JNK activity is significantly enhanced in many cancer cell lines. It has been reported that cell migration behavior caused by polar gene deletion is dependent on JNK signaling, and down-regulating JNK signaling pathway can inhibit cell migration caused by polar gene down-regulation (Figures 2 F, L, and R). In order to investigate whether the loss of Tip60 acetyltransferase activity activates JNK signaling, we used *sd*-Gal4 to examine the activation of *mmp1*, a target gene of JNK signaling pathway, in the disc-wing pocket region of the wings. Loss of Tip60 acetyltransferase activity resulted in a significant upregulation of mmp1 (Figure 4C) compared to the control group (Figure 4A). Wild-type Tip60^{WT} alone does not activate JNK signaling reporter genes (Figure 4B), but inhibits *mmp1* activation caused by loss of Tip60 acetyltransferase activity (Figure 4D). These results indicated that the loss of Tip60 acetyltransferase activity could induce the activation of JNK signaling pathway.

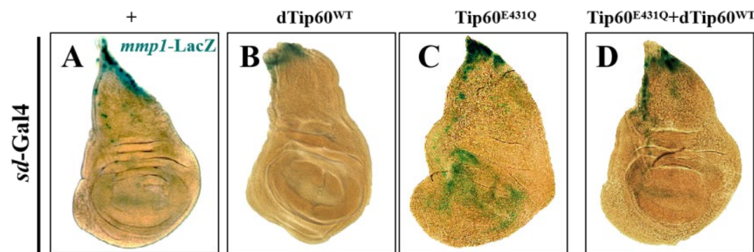

Figure 4: Deletion of Tip60 acetyltransferase activity activated the expression of JNK signaling target genes.

In order to explore the role of Tip60 on the JNK signaling pathway, we conducted genetic host analysis experiments to down-regulate the JNK signaling trunk components dTAK1, hep and bsk, respectively, and observe their changes in cell migration induced by the loss of acetylation function of Tip60. The results showed that *dTAK1-RNAi*, *hep-RNAi* and *bsk-RNAi* could not effectively inhibit cell migration and MMP1 activation induced by Tip60^{E431Q} (Figures 5B-D, J-L and R-T) compared with LacZ expression control (Figures 5A, I and Q). It is proved that Tip60 plays a role in the downstream of Bsk. Under external pressure, JNK phosphorylates two important downstream transcription factors FoxO and Fos, and activates the expression of target genes such as *hid*, *rpr* and *mmp1*, thereby inducing physiological activities such as cell migration and apoptosis. In order to examine which downstream transcription factors of Tip60 affect JNK signaling pathway, we down-regulated *foxo* and *fos*, respectively, and observed their changes in cell migration induced by the loss of Tip60 acetylation function. While *foxo-IR* could not inhibit cell migration phenotype and MMP1 activation (Figures 5E, M, and U), both *fos-IR* and *fos* hybrid mutants (*fos*$^{1/+}$) could inhibit cell migration well (Figures 5F, G, N, O, V, and W). We also transgene human wild type Tip60 (hTip60^{WT}) into *Drosophila* and found that the cell migration induced by the loss of Tip60 acetyltransferase activity in *Drosophila* was well inhibited (Figures 5H, P, and X). These results indicated that Tip60 regulates cell migration through JNK-Fos signaling pathway.

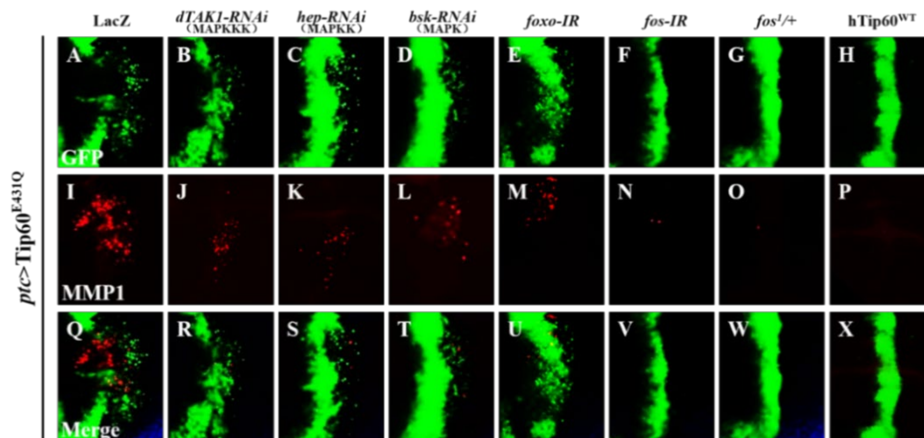

Figure 5: Tip60 regulates cell migration through the JNK-Fos pathway.

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3.4 Tip60 regulates FOS-dependent tumor invasion

The proto oncogene *Ras* can synergistically induce tumorigenesis with the loss of cell polarity. For example, simultaneous expression of persistently activated Ras protein (Ras^{V12}) in *lgl*-mutated clones promotes tumor-like growth in the eye disc (Figure 6C), and malignant proliferating cells invade the abdominal nerve cord (VNC) (Figure 6D). This is a classic model developed by Professor Xu Tian's lab to study the mechanism of tumor invasion in vivo. We found that $Ras^{V12}/lgl^{-/-}$ induced tumor invasion from the adult disc to VNC was inhibited by overexpression of wild type Tip60 (Figures 6F and M), and the size of primary tumors in the adult disc was also partially inhibited (Figure 6E), indicating that Tip60 could effectively inhibit tumor occurrence and invasion caused by $Ras^{V12}/lgl^{-/-}$.

Loss of cell polarity or activation of the cell morphogenetic gene can induce JNK-dependent cell migration and collaboratively induce tumor growth and invasion with Ras^{V12}. Since overexpression of Tip60 can inhibit JNK-dependent cell migration and tumor metastasis, we hypothesized that loss of Tip60 acetyltransferase activity can synergically promote tumor growth and invasion with Ras^{V12}. The results showed that the overexpression of Ras^{V12} only led to the enlargement of the disc volume of the eye, but did not induce tumor occurrence or invasion (Figures 6G, H, and M). However, when $Tip60^{E431Q}$ was expressed with Ras^{V12} at the same time, the disc of adult eye worm developed tumorigenic growth and invaded VNC (anatomical observation 8 days after oviposition) (Figures 6I, J, and M). The synergies between loss of Tip60 acetyltransferase activity and oncogene Ras^{V12} were sufficient to induce tumor growth and invasion. More importantly, *fos* down-regulation can disrupt this synergistic tumor growth and invasion (Figures 6K, L, and M), which is consistent with Fos's reported function of proto-oncogenes.

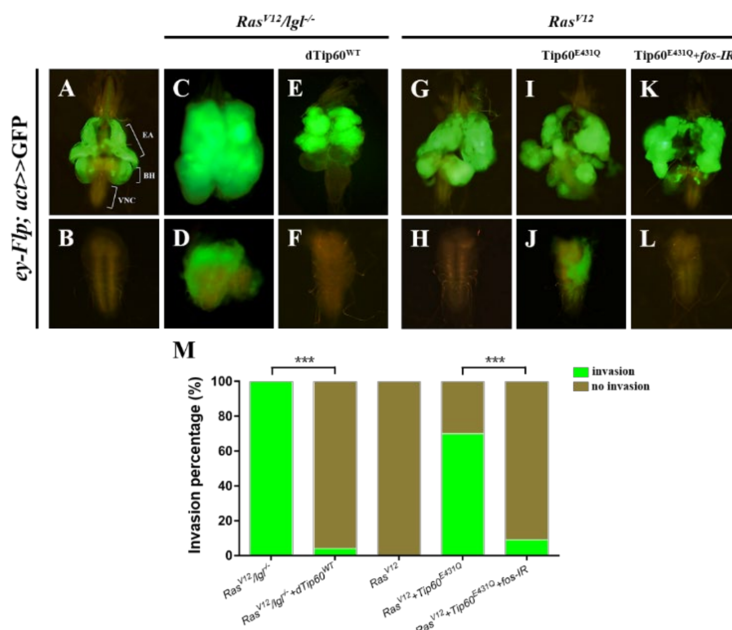

Figure 6: Tip60 is critical for RasV12-induced tumor growth and invasion.

3.5 Overexpression of Tip60 interferes with endogenous JNK-mediated dorsal closure process

The back closure process is another important in vivo model for studying cell migration during *Drosophila* development. JNK signaling is required to regulate the back and chest closure during *Drosophila* development, and the specific downregulation of JNK signaling pathway in the back can produce a back fracture phenotype. We found that overexpression of Tip60 produced a similar phenotype (Figure 7B), while the crack phenotype caused by *pnr*>Tip60 could be further enhanced by the removal of an endogenous JNK (Figure 7C), whereas overexpression of JNK or Fos could partially save the phenotype (Figures 7D and E). The above results fully demonstrated that overexpression of Tip60 in *Drosophila melanogaster* could inhibit the JNK-Fos signaling pathway mediated cell motility, including tumor cell invasion, cell migration and back closure.

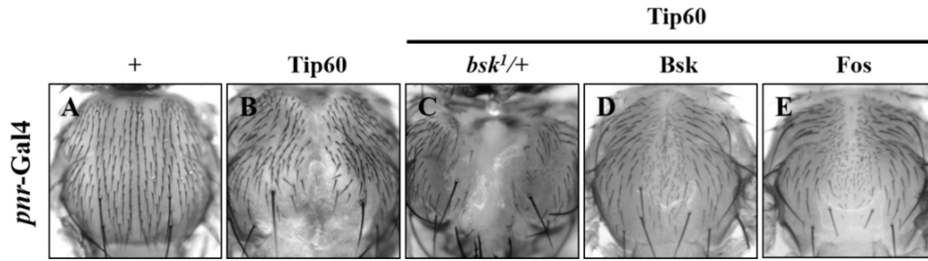

Figure 7: Tip60 was involved in the migration and closure of Drosophila melanogaster back cells.

3.6 Tip60-Fos regulates invasion and migration in human renal carcinoma cells

To test whether Tip60-Fos interactions are evolutionarily conserved? We further investigated the role of human Tip60 and c-Fos in human cancer. To identify cancer cell lines, we first found in the tumor database that Tip60 was less expressed in renal clear cell carcinoma (KIRC) tissues than in para-cancer tissues, and that JNK target genes MMP1, MMP9, and DUSP10 were elevated in cancer tissues. Therefore, we selected human renal clear cell adenocarcinoma cell line 786-O as the mechanism-verified cancer cell line, and transfected c-Fos with GFP label and Tip60 with Flag label in 786-O cells at the same time, so as to directly observe the localization of Tip60 and c-Fos in living cells. Immunofluorescence results showed that in 786-O cell line, c-Fos and Tip60 were well colocalized in the nucleus (marked by blue DAPI) (Figure 8A). Co-immunoprecipitation also showed that c-Fos and Tip60 could bind to each other in 786-O cell line (Figure 8B). WB results showed that wild-type Tip60 significantly promoted the acetylation of Fos in renal carcinoma cells compared with negative controls that overexpressed GFP, while Tip60 mutants with deficient acetyltransferase activity (Tip60^{ATD}) did not (Figure 8C). Down-regulation of Tip60 by RNAi in 786-O cell line activated the expression of MMPs, a downstream migration gene of JNK signaling (Figure 8D) and promoted the phosphorylation of JNK (Figure 8E). The cell migration function experiment also showed that down-regulation of Tip60 significantly promoted the migration ability of 786-O cells, which was inhibited by down-regulation of Fos (Figures 8F and G). These results indicated that Tip60 was highly conserved in regulating JNK-FOS-mediated cell migration from *Drosophila* to *human*.

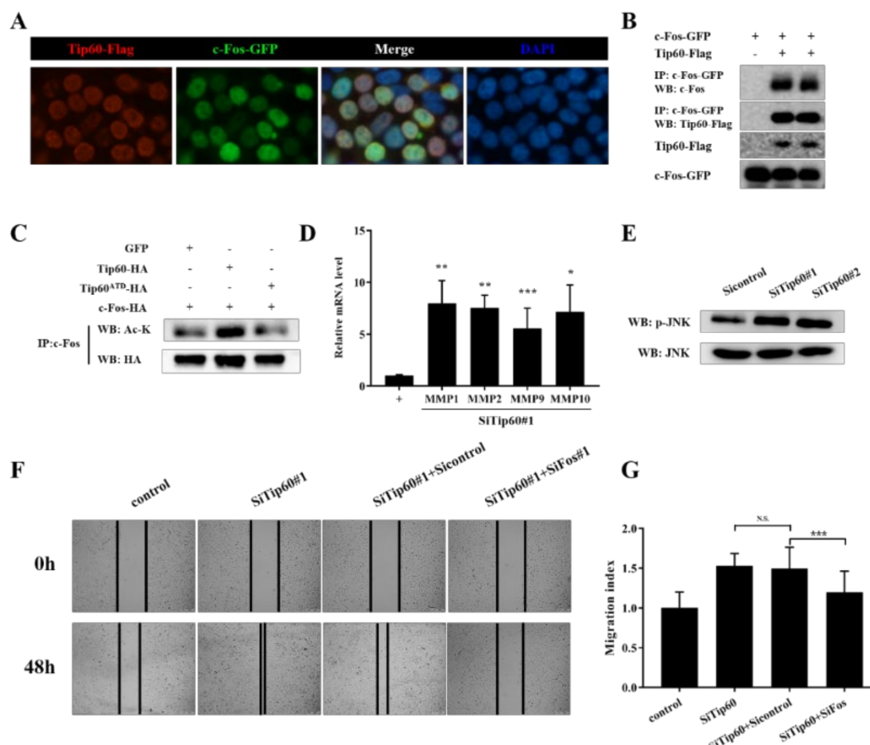

Figure 8: Tip60 regulates JNK-FOS-mediated renal carcinoma 786-O cell invasion.

3.7 Tip60 is involved in the occurrence and development of renal hyaluronic cell carcinoma

Renal parenchymal carcinoma is an adenocarcinoma derived from tubular epithelial cells of the kidney, 85% of which are clear cell carcinomas. c-Fos protein is highly expressed with the increase of KIRC stage, and is closely related to prognosis. TCGA database data analysis showed that the survival rate of KIRC patients with high expression of Tip60 was higher than that of those with low expression of Tip60 (Figure 9A), and Tip60 was low expression in KIRC samples (Figure 9B), indicating that Tip60 played an important role in cancer suppression in the development of KIRC. Immunohistochemical experiments were conducted on 90 renal clear cell carcinoma specimens collected from the Department of Nephrology, Zhuhai People's Hospital. The results showed that in the tissue sections of KIRC patients, the expression of Tip60 protein was mainly located in the nucleus of tumor cells and was lower than the normal level (Figures 9C and D). These results indicated that Tip60 was involved in the occurrence and development of renal hyaluronic cell carcinoma.

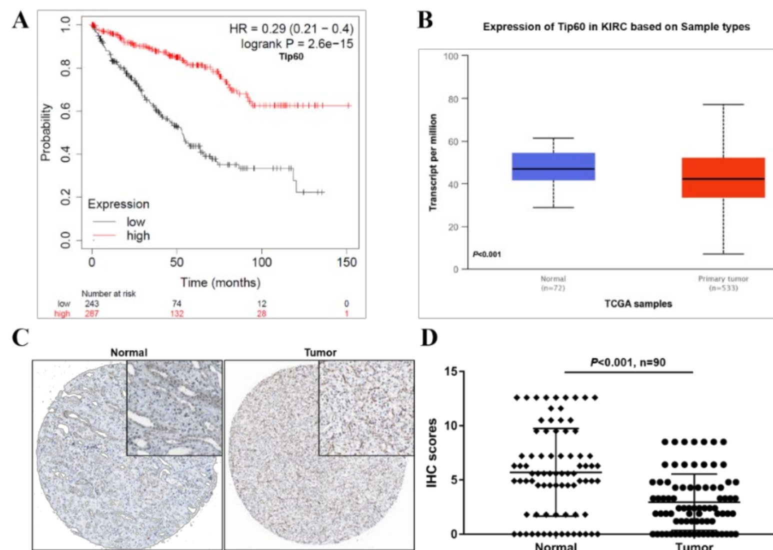

Figure 9: Tip60 inhibits the occurrence and progression of renal clear cell carcinoma.

4. Conclusions

4.1 A new factor regulating cell migration, Tip60, was screened in Drosophila model

By using the genetic advantages of *Drosophila*, we established an animal screening model (*ptc>scrib-RNAi*) based on the migration of disc-line cells to the rear of adult wing worms caused by polar gene deletion, and screened the genes that might be involved in regulating cell migration. After screening, we found that the overexpression of acetyltransferase Tip60 could inhibit cell migration well. Down-regulating *Tip60* or overexpressing mutant Tip60 ($Tip60^{E431Q}$) with loss of acetyltransferase activity can fully induce cell migration and activate the expression of migration molecules such as MMP1.

4.2 The mechanism of cell migration and tumor metastasis induced by loss of Tip60 acetyltransferase activity was explored

In combination with the migration phenotype and molecular markers such as MMP1 and *puc*-LacZ, we found that Tip60 affects the ability of cell migration by regulating JNK signaling. Through genetic experiments, we found that the most important acetyltransferase function of Tip60 affects the JNK signaling pathway. As with *Tip60-RNAi*, overexpressing mutant Tip60 with loss of acetyltransferase activity induced cell migration phenotype and activation of JNK signaling, which could be inhibited by overexpressing normal Tip60. More significantly, just as JNK signaling pathway activation promotes tumor invasion, Tip60 with over expression of acetyltransferase activity deficiency can synergically induce tumor occurrence and invasion with Ras^{V12}.

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4.3 The mechanism of cell migration mediated by JNK signal inhibited by Tip60 was analyzed

We first studied the upstream and downstream relationship between Tip60 and dTAK1, Hep and Bsk through genetic epistatic analysis experiments, and found that Tip60 acted on the downstream regulation of JNK signal in Bsk. By using *ptc*-Gal4 to drive the overexpression of mutant with the deletion of Tip60 acetyltransferase activity, the cell migration phenotype could be generated. We down-regulated common transcription factors downstream of JNK to inhibit this phenotype, and found that only the RNAi and mutant of *fos* could inhibit the cell migration phenotype caused by $Tip60^{E431Q}$. At the same time, we also verified the relationship between Tip60 and Fos in *Drosophila* tumor invasion model. In order to further verify whether Tip60 regulates JNK signaling pathway through Fos mediation, we conducted cell and molecular experiments. We observed that Tip60 and Fos co-localized in the nucleus, and Tip60 was able to acetylate Fos. The binding of Fos to Jun after acetylation is reduced, thus inhibiting the cell migration caused by the activation of JNK signaling pathway.

4.4 The conserved effect of Tip60-Fos on cell migration and tumor invasion was verified

In order to verify the conserved effect of *Drosophila* Tip60-Fos interaction in humans, we introduced transgenic *Drosophila* lines to express human Tip60 ($hTip60^{WT}$) in this project. We observed that cell migration and tumor invasion phenotypes caused by Tip60 inactivation in *Drosophila* could be saved by human Tip60. At the same time, we found that human Tip60 can also inhibit a series of phenotypes caused by JNK signal activation, which is consistent with the function of *Drosophila* Tip60. Further cancer cell validation experiments showed that Tip60 and c-Fos co-localized in renal carcinoma 786-O cell line. The results of molecular experiments showed that Tip60 and c-Fos could bind to each other and acetylate them. At present, in various cancers such as colon cancer, breast cancer and prostate cancer, the abnormal function of Tip60 may play a role in promoting or inhibiting tumor, but its specific molecular mechanism is not clear. In combination with the regulatory relationship of Tip60 on JNK signaling found in fruit flies, we plan to verify the role of Tip60-Fos in the occurrence and invasion of kidney cancer using renal hyaluronic cell cancer cell lines and samples of renal cancer patients, and analyze the association between the expression level of Tip60 and the survival rate of patients with bioinformatics.

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