Importin α1 mediates Yorkie nuclear import via N-terminal non-canonical nuclear localization signal

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Running Title: Importin α1 mediates Yorkie nuclear import

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Abstract

The Hippo signaling pathway controls organ size by orchestrating cell proliferation and apoptosis. When the Hippo pathway was inactivated, the transcriptional co-activator Yorkie translocates into the nucleus and forms complex with transcription factor Scalloped to promote the expression of Hippo pathway target genes. Therefore, the nuclear translocation of Yorkie is a critical step in Hippo signaling. Here, we provide evidence that the N-terminal 1-55 amino acids of Yorkie, especially R15, were essential for its nuclear localization. By mass spectrometry and biochemistry analyses, we found that Importin α1 can directly interact with Yorkie N-terminus and drive Yorkie into the nucleus. Further experiments show that the upstream component Hippo can inhibit Importin α1-mediated Yorkie nuclear import. Taken together, we identify a potential nuclear localization signal at the N-terminal end of Yorkie as well as a critical role of Importin α1 in Yorkie nuclear import.

Introduction

The mechanisms by which organ size is controlled during development have long been an intriguing question. Extrinsic environmental factors and intrinsic signals function cooperatively in organ development (1). The Hippo pathway, initially discovered in Drosophila by genetic screens, restricts organ size through inhibiting cell growth and promoting cell death (2,3). In Drosophila, the core of the pathway is a kinase cassette including kinases Hippo and Warts, which triggers the phosphorylation of transcription coactivator Yorkie (Yki) (4-9). In the absence of the suppression from Hippo signaling, Yki translocates into nuclei and hence associates with transcription factor Scalloped (Sd) (10-12). As a result, the Yki-Sd complex induces the expression of genes, including diap1 (Death-associated inhibitor of apoptosis 1), cyclineE and bantam microRNA, to regulate cell proliferation and apoptosis (13-15).

The core components of Drosophila Hippo pathway are highly conserved in mammals. Although the regulation of cytoplasm-to-nucleus translocation of Yki or its mammalian homologue YAP (Yes-associated protein) is a fundamental step in Hippo signaling, little was known about the
underlying mechanisms. The nuclear localization of proteins is mediated by two mechanisms. One is passive diffusion through the nuclear pore complex (NPC), which only suitable for proteins smaller than approximately 40-50 kDa (16,17). The second one is an energy-dependent process, which allows proteins that contain a nuclear localization signal (NLS) to enter the nucleus (18,19). NLSs are short peptide motifs that mediate the nuclear import of proteins. Classical NLSs always have one or two clusters of basic amino acid residues, and two clusters always separated by a 10-12 amino acid linker, defined as (K/R)(K/R)X10-12(K/R)3/5 (20-23). However, a number of experimentally defined NLSs do not match the consensus sequences, sometimes they contain only one or two lysine or arginine (24-26).

In mammalian systems, NLSs are usually recognized by the heterodimeric receptor proteins Importin α/β. Importin-substrate complexes translocate into the nucleus through the NPC, and with the help of RanGTP/RanGDP, the Importin α can recycle back to the cytoplasm(27,28). Crystallographic approaches and mutational analysis reveals that the cNLS-binding domain of Importin α is located within a shallow groove on the concave face, which comprised of ten armadillo (ARM) repeats. The primary binding site for NLSs spans ARM repeats 1–4 (the major site), and a secondary site (the minor site) spans ARM repeats 6–8(29-32). In Drosophila melanogaster, Importin α family contains Importin α1, Importin α2 and Importin α3 (33-35). Importin α1 and α2 play distinct role in male and female gametogenesis (36,37); Importin α3 is required for larval survival, structure development and completion of oogenesis (35).

In this study, we demonstrate that the N-terminal 1-55 amino acids of Yki may serve as its NLS and is essential for Yki’s function and localization. We also identify Importin α1 as a mediator of Yki nuclear translocation using mass spectrometry analysis. Furthermore, we show that the amino acid R15 of Yki is a critical site for Yki nuclear import, which is regulated by upstream core kinase Hippo. Therefore, our observations demonstrate the functional and physical interactions between Yki and Importin α1.

Materials and methods

Cloning, Drosophila stocks and Genetics

Importin α1, α2, α3, β cDNAs were amplified by PCR and introduced into pUAST-HA or pUAST-Flag vector. Yki point mutations and deletions were generated by PCR based site directed mutation. YkiN mutant form was produced by gene synthesis (Generay). All plasmids were verified by DNA sequencing. Transgenic flies expressing α1 or β were generated by injections. A pUAST vector with attB sequence was used to make pUAST-attB-Yki, pUAST-attB-YkiM2 and Yki truncation form flies. Importin α1 mutant fly is a deficiency line from Bloomington Drosophila Stock Center (stock number 25396), which removes the complete importin α1 gene. Importin α1 RNAi is from Bloomington (27523).The following transgenes were used in this study: ex-lacZ, hh-Gal4, GMR-Gal4, UAS-Yki, hpoBF33, YkiB5, which has been described previously (10,38,39). All the flies were cultured at 25°C.

Cell culture, Transfection, co-immunoprecipitation, western blot and luciferase reporter assays

S2 cells were maintained in Drosophila Schneider’s Medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. The cells were incubated at 25°C in a humidified air atmosphere.

Plasmid transfection was carried out using Lipofectamine (Invitrogen), according to the manufacturer’s instructions. For all the transfection experiments, an ubiquitin-Gal4 construct was co-transfected with the pUAST expression vectors. For immunoprecipitation, after 48h of expression, cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1.5mM EDTA, 10 mM NaF, 1 mM Na3VO4) supplemented with Protease Inhibitor Cocktail (Sigma). Cell lysates were pre-cleared by Protein A beads (GE Healthcare) and then incubated with indicated antibodies. Western blot analyses were performed according to standard protocols as previously described. The following antibodies were used in the immunoprecipitation or Western blot analyses: mouse anti-HA (Sigma), mouse anti-Flag (Sigma), mouse anti-Myc (Sigma), and mouse anti-HRP (Santa cruz) using dilutions of 1:5000 for all antibodies.
For the luciferase reporter assay, the 3xSd2-Luc reporter has been previously described (10). The luciferase assay was performed using the Dual Luciferase Assay System (Promega).

**Immunofluorescence staining**

S2 cells and imaginal discs were fixed in 4% formaldehyde (Sigma) and washed three times in PBS supplemented with 0.1% Triton X-100 (PBS-T). The cells and discs were incubated in the primary antibody diluted in PBS-T for 1 h at room temperature, followed by three washes with PBS-T. After a same incubation and washing with a second antibody, cells and discs were mounted in PBS/glycerol medium with DAPI. Primary antibodies used include: mouse anti-Myc (1:500), rabbit anti-Flag (1:200), rabbit anti-HA (1:200), rat anti-Ci (1:250, DSHB), mouse anti-lacZ (1:500, Invitrogen).

**Mass Spectrometry**

For each sample, ten 10 cm dishes of S2 cells were collected and lysed in 8 ml lysis buffer (Tris HCL pH 8.0, 50 mM, NaCl 100 mM, NaF 10 mM, Na3VO4 1 mM, EDTA 1.5 mM, NP-40 1%, glycerol 10% supplied with protease inhibitor cocktail). Then centrifuge at 14,000 rpm for 20 minutes at 4°C and take the supernatant as storage. GST-tagged YkiN 1-55 amino acid plasmid was made for the protein purification. 0.5 mg protein was coupled with 500 ml Affi-Gel10 beads for 4 hours at 4°C. The beads were washed with cold lysis buffer for 3 times and mixed with the cell supernatant at 4°C for 2 hours on a rolling mixer. After 3 times washing, the beads were sent directly for MS analysis.

**BrdU incorporation**

*Drosophila* eye discs were dissected and incubated in PBS with 75 µg/ml BrdU for 35 minutes, then fixed in 5% formaldehyde for 45 minutes. DNAs were denatured by treating in 3M HCl for 30 minutes. Discs were washed 3 times, and then treated as described immune staining. Mouse anti-BrdU (Sigma) was used as 1:500.

**Microscopy image acquisition and Statistics**

Fluorescent microscopy was performed on a Leica LAS SP8 confocal microscope; confocal images were obtained using the Leica AF Lite system. The cells were taken photos using a 60× objective and discs were using a 20× objective. All the data were expressed as the mean standard error of the mean (SEM) and were analyzed using Student’s t-test. The results were considered statistically significant if p<0.05.

**Results**

*N-terminus of Yki is essential for its function and nuclear localization*

To explore the underlying mechanism of how Yki translocates into the nucleus, we first performed cell-based immunostaining assays. Majority of overexpressed wild-type Yki maintain in the cytoplasm, while coexpression of Sd caused significant Yki nuclear translocation (Figure 1A – C and H). Surprisingly, a truncation of Yki (YkiN) that lacks N-terminal 1-55 amino acids mainly distributes in the cytoplasm and near the cell membrane, and even Sd overexpression was not able to drive YkiN into nucleus (Figure 1D-E and H). However, the missing part (YkiN) exhibits a dispersive distribution in S2 cells and cannot response to Sd (Figure 1F-G and H).

To dissect the function of YkiN on Yki cellular localization and activity, a dual luciferase assay was performed using S2 cells (Schneider 2 cells). Co-expression of Yki full-length with Sd synergistically activates the luciferase reporter gene (3xSd2-Luc), while co-expression of YkiN or YkiN with Sd failed to induce dramatic activation (Figure 1I), indicating that N-terminus of Yki is necessary but not sufficient for Yki’s activity in vitro. To confirm this finding in vivo, transgenic flies expressing Yki variants were generated using the phiC31 integration system, which ensures that the transgenes are expressed at an equal level. Overexpression of Yki full-length by eye-specific driver GMR (glass multiple reporter)-Gal4 caused an overgrowth phenotype (Figure 1J-K). As expected, overexpressing YkiN or YkiN with Sd failed to induce dramatic activation (Figure 1I), indicating that N-terminus of Yki is necessary but not sufficient for Yki’s activity in vitro. To confirm this finding in vivo, transgenic flies expressing Yki variants were generated using the phiC31 integration system, which ensures that the transgenes are expressed at an equal level. Overexpression of Yki full-length by eye-specific driver GMR (glass multiple reporter)-Gal4 caused an overgrowth phenotype (Figure 1J-K). As expected, overexpressing YkiN or YkiN with Sd failed to induce dramatic activation (Figure 1I). In addition, the activity change of the Hippo pathway was examined by detecting the level of ex (expanded)-lacZ, an enhancer trap for ex. Neither YkiN nor YkiN that expressed by wing-specific driver hh-Gal4 could induce a significant upregulation of ex-lacZ as Yki full-length (Figure 1O-R’). Conclusively, N-terminus
of Yki is necessary but not sufficient for Yki’s function in Hippo signaling.

Yet, what makes YkiN so important in Sd-mediated Yki cytoplasmic-nuclear shuttle? Generally, the nuclear localization of proteins over 50 kDa is mediated by a ribosomal synthesis and establishment of the concentration gradient between the cytoplasm and nucleus, which allows proteins that contain a NLS to enter the nucleus (18,19). We speculated that Yki might contain a NLS to enter the nucleus between the cytoplasm and nucleus, which allows establishment of the concentration gradient 50 kDa is mediated by an ribosomal synthesis and mediated Yki cytoplasmic-nuclear shuttle?function in Hippo signaling.

To verify whether Importin α1 is a bona fide Yki regulator in vivo, we inducted an importin α1 null fly line. Overexpression of UAS-Yki from the posterior to the morphogenetic furrow (MF) using the GMR-Gal4 driver (GMR-Yki) resulted in enlarged eyes, while expression of Yki in importin α1 hypomorphic mutant flies (α1+/−) led to smaller and smooth eyes (Figure 3A-C’). BrdU and Diap1, markers of proliferation and anti-apoptosis, were increased dramatically when Yki was overexpressed while decreased in α1+/− background (Figure 3A’-C’). Collectively, the inactivation of Importin α1 suppressed Yki’s function. To strengthen this conclusion, the expression level of Diap1, which reflects Yki activity, was examined in Importin α1 mosaic clones. In α1null MARCM (Mosaic analysis with a repressible cell marker) clones, Yki overexpression-induced upregulation of Diap1 is significantly blocked (Figure 3D-F”). Importantly, the sizes of α1null clones were small, even when Yki was overexpressed, indicating a proliferative disadvantage of α1null clones. However, considered the large number of Importin α1 targets, there might be other collaborating transcription factors, such as E2F or Mad, which also shuttles, to join in this processes. Furthermore, we analyzed the genetic epistasis between hpo (hippo) and Importin α1. In hpo mutant clones induced by the MARCM system, the ablation of hpo resulted in Figure 2E-H and K, upon α1 coexpression, majority of Yki translocate into the nucleus. Of note, α2 or α3 coexpression did not cause a similar change, consistent with the immunoprecipitation results in Figure 2C. In addition, α1 only changes the localization of YkiN but not YkiΔN (Figure 2I-J’ and K). Importantly, a clear and profound nuclear import of YkiN in vivo can be detected (Figure 2L-L’). However, no dramatic nuclear localization of Yki full-length upon α1 expression was observed in vivo (Data not show). We speculated that Yki full-length is functional in vivo and its nuclear translocation is tightly regulated by multiple factors and mechanisms. In toto, we conclude that Importin α1 drives Yki nuclear localization via binding to its N-terminus.

Importin α1 is indispensable for Hippo signaling in vivo

To dissect the regulatory mechanism of Yki cytoplasmic-nuclear translocation, we perform a mass spectrometry (MS) analysis using purified GST-tagged YkiN protein as bait. Two nucleoporin, Importin α1 and pen (Importin α2), have been identified (Figure 2A-B). Since YkiN might function as a NLS (Figure 1), we deduce that the Yki cytoplasmic-nuclear translocation may be through nucleoporin complex. To test this hypothesis, we constructed HA-tagged α1 (α1), Importin α2 (α2), and Importin α3 (α3) for co-immunoprecipitation assay. Interestingly, only α1 directly interacted with Yki in two directions (Figure 2C). To narrow down the binding region of Yki-α1, we detected the association between YkiN/Yki Δ N and α1. YkiN has a strong association with α1 in two directions, whereas no distinct interaction of YkiΔN-α1 was observed (Figure 2D). It is feasible to consider that Importin α1 drives Yki nuclear translocation. As shown in...
tumor-like overgrowth and elevated the protein levels of Ex, while these changes can be markedly suppressed by Importin αRNAi (Figure 3G-I’’’). These observations suggest that Importin α1 acts downstream of hpo to influence Hippo signaling.

**Yki R15 is critical for Yki’s association with Importin α1 and its cellular localization**

We have showed the indispensable roles of Yki’s N-terminus and Importin α1 in nuclear translocation of Yki. To dissect how Importin α1 regulates Yki’s function, we first mapped the N-terminus of Yki. It has been reported that the classic NLS motif always contains several Lysine and Arginine repeat, but some untypical NLS may only contains one or two Lysine or Arginine (20). No classical NLS sequence was found in YkiN, but there are three Lysine and one Arginine residues (Lys8, Arg15, Lys40, and Lys52) that could be important for nuclear import (Figure 4A). YkiN variants that contain point mutation of these sites together or individually were generated and examined (Figure 4A). As expected, YkiNM, which contains four mutations together, failed to interact with Importin α1 (Figure 4B), and localized mainly in the cytoplasm with an accumulation near the cell and nuclear membranes (Figure 4E-E’). Strikingly, YkiNM2 that bears a single point mutation of R15A blocks Yki-Importin α1 association in two directions, whereas the other three single mutations had no effects on Yki-Importin α1 interaction (Figure 4A-B). Also, YkiNM2, but not other three, exhibited a similar localization as YkiNM, which failed to respond to Importin α1 (Figure 4F-J).

However, we noticed a mobility shift of Yki mutants bearing R15A mutation (YkiNM2) (Figure 4B). We speculated that the change of R15 might induce changes of Ser or Thr phosphorylation status in YkiN. We classified thirteen Ser and Thr on YkiN into groups and generated mutants accordingly and identified the point mutation combination in YkiNM5 blocked the shift band caused by R15A mutation (Figure 4A and 4C). Subsequently, we tested if phosphorylation status of these sites influences YkiN localization using YkiNM6 mutant, which contains same Ser/Thr mutations as YkiNM5 but lacks R15 mutation (Figure 4A and 4C). Unlike YkiNM5, YkiNM6 still interacts with Importin α1 and is able to translocate into the nucleus like wild-type YkiN (Figure 4J). Collectively, the mobility shift of Yki mutants bearing R15A mutation does not affect its nuclear translocation.

To test the function of Yki R15, the differences among Yki and YkiM2 (full-length Yki bearing R15A mutation) in the interaction with Importin α1 and cellular localization were examined. Consistently, the mutation of R15 in full-length Yki also disrupts its association with Importin α1 and showed a similar cellular localization as YkiNM2 (Figure 5A and B-F). We then checked the functional difference in vivo using transgenic fly expressing equal level Yki and YkiM2 in MARCM system. Because Yki was an important factor in growth control, yki null clones have very small size and obviously decreased Diap1 level (Figure 5G-H’’’). Upon overexpression of Yki, the clone size and Diap1 level were dramatically increased (Figure 5I-I’’’), suggested a high activity of overexpressed wild-type Yki. However, YkiM2 showed no influence on the clone size and Diap1 level for yki null clones, (Figure 5J-J’’’), indicating YkiM2 exhibits functional defect and hence was not able to rescue yki mutant clones. This compelling evidence strongly supports our finding that amino acid residue R15 of Yki is important for the function of Yki in vivo. In other words, the localization of Yki and/or its binding ability with Importin α1 is critical for the function of Yki.

**Hippo signaling regulates Yki localization via impairing the binding between Yki and Importin α1**

The Hippo pathway regulates the activity of Yki by tight control of its cytoplasmic-nuclear shuttle through inducing physical interaction with 14-3-3 proteins (40). Here raise the question that whether importin α1 has relationship with 14-3-3, or whether they affect each other? As shown in Figure 6A, 14-3-3 interacts with Yki△N, but not YkiN. In addition, the interaction between Yki and Importin α1 was not affected by 14-3-3 coexpression (Figure 6B). These observations suggest that Importin α1 and 14-3-3 regulate Yki cellular localization via different mechanisms by binding different regions.

The role of Hpo kinase in Importin α1-mediated Yki localization regulation was also explored. As shown in Figure 6B, the binding between Yki and Importin α1 was attenuated,
when Hpo was coexpressed. YkiS168A is reported to escape Hippo signaling-mediated regulation and have a higher level of nuclear localization (41,42). Indeed, YkiS168A exhibits a stronger association with Importin α and coexpression of Hpo failed to affect the binding (Figure 6C). Moreover, in competitive binding assay, Hpo kinase activity is required for regulating Yki-Importin α association (Figure 6D). As shown in Figure 6E-I, Hpo causes Yki retardation in the cytoplasm even when Importin α is coexpressed. These findings indicated a dominant role of Hpo in Importin α-dependent Yki nuclear trans-localization. Moreover, we tested the role of Ran in Yki localization regulation. As shown in Fig 6J, the interaction between Yki and Importin α decreased upon RanQ69L (Ran-GTP) addition, while the interaction was not affected by addition of RanT24N (Ran-GDP), suggesting that the binding between Yki and Importin α is Ran-GTP sensitive.

Discussion

It has been reported that the Hippo pathway plays essential roles in organ size control, stem cell maintenance, tissue homeostasis and repair (2,43-45). The nuclear import of Yki, or its mammalian homolog Yap, is a critical step for Hippo signaling transduction. However, little was known about the mechanism of this process. Based on the MS screen results, we demonstrated that Importin α might be involved in Yki nuclear translocation through interacting with N terminus of Yki. The nuclear import of most proteins by Importin α requires a classical NLS (46,47). We proposed that, although Yki do not contain such motif, the N terminal 1-55 amino acids of Yki, especially R15, act as an NLS to interact with Importin α and mediate the nuclear translocation of Yki. Furthermore, by testing the regulation from upstream kinase Hpo, the close relationship between Importin α and Hippo pathway was revealed (Figure 7).

Hippo signaling pathway suppresses Yki nuclear localization by mediating Yki phosphorylation, which promotes the binding between Yki and 14-3-3 proteins to detain Yki in the cytoplasm (40,41). Here raise the question that whether importin α has relationship with 14-3-3 or whether two proteins affect each other. First, we showed that the interaction of Yki and 14-3-3 is independent on Yki N-terminal region. Subsequently, we demonstrated that 14-3-3 did not affect the binding between Yki and Importin α. We supposed that Importin α and 14-3-3 regulate Yki nuclear trans-localization through different mechanisms.

According to the immunostaining data in S2 cells (Figure 2), Importin α can promote the nuclear localization of Yki full-length and YkiN dramatically. However, when performing the same experiment in Drosophila imaginal discs, we only observed a similar trend of nuclear localization of YkiN, but not that of Yki full-length. The explanation for this observation would be that the process of Yki nuclear-cytoplasmic shuttle was tightly regulated by multiple mechanisms.

As the data shown in Figure 1D-E, deleting the N-terminal region of Yki not only prevents Yki nuclear translocation but also enhances Yki cortical localization near the outer cell membrane. One possibility might be that YkiN can bind factors other than Importin α to prevent membrane association. In MS analysis, we discovered multiple candidates of possible factors. Further experiments are needed to explore the underlying mechanism regarding this matter.

An evolutionally conserved function of Yki in growth control has been reported. To determine whether the function of Importin α is conserved, the human homolog of Importin α, KPNA6 was cloned. However, when co-expressed Yap and KPNA6 in 293T cells or MCF10A cells, Yap did not show higher levels of nuclear localization. In addition, no physical interaction was detected in 293T cells (data not shown). It is reported that there are more than twenty Importins exist in human. Therefore, Yap nuclear translocation may also be mediated other Importin members or by additional mechanisms (27,48).

Since the nuclear localization of Yap is a remarkable marker in many kinds of human cancers, it will be meaningfully to investigate the Yap nuclear import regulation. Therefore, further studies on YAP trans-localization might help to solve the puzzle.

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions
Author Contributions: S.W., Y.L. M-X.Y. and L.Z. conceived and designed the experiments; S.M., Y.L., M-X.Y., C.W. performed the experiments; S.M., Y.L., M-X.Y., C.W., L.Z. analyzed the data; W.W., J.H., WQ.W., L.H., L.G. contributed regents/materials; S.M., M-X.Y and L.Z wrote the paper.
References

1. Dong, J., Feldmann, G., Huang, J., Wu, S., Zhang, N., Comerford, S. A., Gayyed, M. F., Anders, R. A., Maitra, A., and Pan, D. (2007) Elucidation of a universal size-control mechanism in Drosophila and mammals. Cell 130, 1120-1133

2. Yin, M., and Zhang, L. (2011) Hippo signaling: a hub of growth control, tumor suppression and pluripotency maintenance. Journal of genetics and genomics = Yi chuan xue bao 38, 471-481

3. Yin, M. X., and Zhang, L. (2015) Hippo signaling in epithelial stem cells. Acta Biochim Biophys Sin (Shanghai) 47, 39-45

4. Harvey, K. F., Pfleger, C. M., and Hariharan, I. K. (2003) The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. Cell 114, 457-467

5. Wu, S., Huang, J., Dong, J., and Pan, D. (2003) hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. Cell 114, 445-456

6. Pantalacci, S., Tapon, N., and Leopold, P. (2003) The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. Nature cell biology 5, 921-927

7. Udan, R. S., Kango-Singh, M., Nolo, R., Tao, C., and Halder, G. (2003) Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nature cell biology 5, 914-920

8. Xu, T., Wang, W., Zhang, S., Stewart, R. A., and Yu, W. (1995) Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase. Development 121, 1053-1063

9. Justice, R. W., Zilian, O., Woods, D. F., Noll, M., and Bryant, P. J. (1995) The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes & development 9, 534-546

10. Zhang, L., Ren, F., Zhang, Q., Chen, Y., Wang, B., and Jiang, J. (2008) The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control. Developmental cell 14, 377-387

11. Goulev, Y., Fauny, J. D., Gonzalez-Marti, B., Flagiello, D., Silber, J., and Zider, A. (2008) SCALLOPED interacts with YORKIE, the nuclear effector of the hippo tumor-suppressor pathway in Drosophila. Current biology : CB 18, 435-441

12. Wu, S., Liu, Y., Zheng, Y., Dong, J., and Pan, D. (2008) The TEAD/TEF family protein Scalloped mediates transcriptional output of the Hippo growth-regulatory pathway. Developmental cell 14, 388-398

13. Nolo, R., Morrison, C. M., Tao, C., Zhang, X., and Halder, G. (2006) The bantam microRNA is a target of the hippo tumor-suppressor pathway. Current biology : CB 16, 1895-1904

14. Thompson, B. J., and Cohen, S. M. (2006) The Hippo pathway regulates the bantam microRNA to control cell proliferation and apoptosis in Drosophila. Cell 126, 767-774

15. Peng, H. W., Slattery, M., and Mann, R. S. (2009) Transcription factor choice in the Hippo signaling pathway: homothorax and yorkie regulation of the microRNA bantam in the progenitor domain of the Drosophila eye imaginal disc. Genes & development 23, 2307-2319

16. Tran, E. J., and Wente, S. R. (2006) Dynamic nuclear pore complexes: life on the edge. Cell 125, 1041-1053

17. Macara, I. G. (2001) Transport into and out of the nucleus. Microbiology and molecular biology reviews : MMBR 65, 570-594, table of contents

18. Gorlich, D., Pante, N., Kutay, U., Aebi, U., and Bischoff, F. R. (1996) Identification of different roles for RanGDP and RanGTP in nuclear protein import. The EMBO journal 15, 5584-5594

19. Jakel, S., and Gorlich, D. (1998) Importin beta, transportin, RanBPS and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. The EMBO journal 17, 4491-4502
20. Kosugi, S., Hasebe, M., Matsumura, N., Takashima, H., Miyamoto-Sato, E., Tomita, M., and Yanagawa, H. (2009) Six classes of nuclear localization signals specific to different binding grooves of importin alpha. *The Journal of biological chemistry* **284**, 478-485
21. Lange, A., Mills, R. E., Lange, C. J., Stewart, M., Devine, S. E., and Corbett, A. H. (2007) Classical nuclear localization signals: definition, function, and interaction with importin alpha. *The Journal of biological chemistry* **282**, 5101-5105
22. Robbins, J., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1991) Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* **64**, 615-623
23. Dingwall, C., and Laskey, R. A. (1991) Nuclear targeting sequences—a consensus? *Trends in biochemical sciences* **16**, 478-481
24. Fontes, M. R., Teh, T., Toth, G., John, A., Pavo, I., Jans, D. A., and Kobe, B. (2003) Role of flanking sequences and phosphorylation in the recognition of the simian-virus-40 large T-antigen nuclear localization sequences by importin-alpha. *The Biochemical journal* **375**, 339-349
25. Hodel, M. R., Corbett, A. H., and Hodel, A. E. (2001) Dissection of a nuclear localization signal. *The Journal of biological chemistry* **276**, 1317-1325
26. Makkerh, J. P., Dingwall, C., and Laskey, R. A. (1996) Comparative mutagenesis of nuclear localization signals reveals the importance of neutral and acidic amino acids. *Current biology : CB* **6**, 1025-1027
27. Wagstaff, K. M., and Jans, D. A. (2009) Importins and beyond: non-conventional nuclear transport mechanisms. *Traffic* **10**, 1188-1198
28. Stewart, M. (2007) Molecular mechanism of the nuclear protein import cycle. *Nature reviews. Molecular cell biology* **8**, 195-208
29. Conti, E., Uy, M., Leighton, L., Blobel, G., and Kuriyan, J. (1998) Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin alpha. *Cell* **94**, 193-204
30. Conti, E., and Kuriyan, J. (2000) Crystallographic analysis of the specific yet versatile recognition of distinct nuclear localization signals by karyopherin alpha. *Structure* **8**, 329-338
31. Fontes, M. R., Teh, T., Jans, D., Brinkworth, R. I., and Kobe, B. (2003) Structural basis for the specificity of bipartite nuclear localization sequence binding by importin-alpha. *The Journal of biological chemistry* **278**, 27981-27987
32. Marfori, M., Lonhienne, T. G., Forwood, J. K., and Kobe, B. (2012) Structural basis of high-affinity nuclear localization signal interactions with importin-alpha. *Traffic* **13**, 532-548
33. Torok, I., Strand, D., Schmitt, R., Tick, G., Torok, T., Kiss, I., and Mechler, B. M. (1995) The overgrown hematopoietic organs-31 tumor suppressor gene of Drosophila encodes an Importin-like protein accumulating in the nucleus at the onset of mitosis. *The Journal of cell biology* **129**, 1473-1489
34. Kussel, P., and Frasch, M. (1995) Pendulin, a Drosophila protein with cell cycle-dependent nuclear localization, is required for normal cell proliferation. *The Journal of cell biology* **129**, 1491-1507
35. Mathe, E., Bates, H., Huikeshoven, H., Deak, P., Glover, D. M., and Cotterill, S. (2000) Importin-alpha3 is required at multiple stages of Drosophila development and has a role in the completion of oogenesis. *Developmental biology* **223**, 307-322
36. Mason, D. A., Fleming, R. J., and Goldfarb, D. S. (2002) Drosophila melanogaster importin alpha1 and alpha3 can replace importin alpha2 during spermatogenesis but not oogenesis. *Genetics* **161**, 157-170
37. Gorjanacz, M., Adam, G., Torok, I., Mechler, B. M., Szlanka, T., and Kiss, I. (2002) Importin-alpha 2 is critically required for the assembly of ring canals during Drosophila oogenesis. *Developmental biology* **251**, 271-282

38. Huang, H. L., Wang, S., Yin, M. X., Dong, L., Wang, C., Wu, W., Lu, Y., Feng, M., Dai, C., Guo, X., Li, L., Zhao, B., Zhou, Z., Ji, H., Jiang, J., Zhao, Y., Liu, X. Y., and Zhang, L. (2013) Par-1 regulates tissue growth by influencing hippo phosphorylation status and hippo-salvador association. *PLoS Biol* **11**, e1001620

39. Huang, J., Wu, S., Barrera, J., Matthews, K., and Pan, D. (2005) The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. *Cell* **122**, 421-434

40. Ren, F., Zhang, L., and Jiang, J. (2010) Hippo signaling regulates Yorkie nuclear localization and activity through 14-3-3 dependent and independent mechanisms. *Developmental biology* **337**, 303-312

41. Oh, H., and Irvine, K. D. (2008) In vivo regulation of Yorkie phosphorylation and localization. *Development* **135**, 1081-1088

42. Oh, H., and Irvine, K. D. (2009) In vivo analysis of Yorkie phosphorylation sites. *Oncogene* **28**, 1916-1927

43. Zhao, B., Li, L., Lei, Q., and Guan, K. L. (2010) The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. *Genes & development* **24**, 862-874

44. Zhao, B., Tumaneng, K., and Guan, K. L. (2011) The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. *Nature cell biology* **13**, 877-883

45. Pan, D. (2010) The hippo signaling pathway in development and cancer. *Developmental cell* **19**, 491-505

46. Sorokin, A. V., Kim, E. R., and Ovchinnikov, L. P. (2007) Nucleocytoplasmic transport of proteins. *Biochemistry. Biokhimiia* **72**, 1439-1457

47. Poon, I. K., and Jans, D. A. (2005) Regulation of nuclear transport: central role in development and transformation? *Traffic* **6**, 173-186

48. Goldfarb, D. S., Corbett, A. H., Mason, D. A., Harreman, M. T., and Adam, S. A. (2004) Importin alpha: a multipurpose nuclear-transport receptor. *Trends in cell biology* **14**, 505-514

**Figure Legends**

**Figure 1. N-terminus of Yki is essential for its nuclear localization and function**

(A) A schematic diagram of Yki and the region corresponding to YkiN/Yki△N. Yki contains two WW domains. YkiN contains amino acids 1 to 55, and Yki△N contains amino acids 56 to 418. (B-G) S2 cells expressing Myc-Yki (B-C), Myc-YkiN (D-E), Myc-Yki△N (F-G) with or without HA-Sd were immunostained with anti-Myc (green) or anti-HA (red) antibodies. Nuclei are marked by DAPI (blue). (H) Cells with different nucleocytoplasmic distributions of Myc-tagged Yki or Yki truncations were counted. A total of 100 cells were counted for each case. The y axis indicates the percentage of cells in each category. (I) The transcription activitis of Yki, YkiN, Yki△N, and nlsYki△N with or without coexpression of Sd in *vivo*. S2 cells were transfected with the indicated constructs and the reporter genes. 48 hours post-transfection, cell lysates were harvested and subjected to a dual luciferase assay. All of these data were represented as the mean±SEM. **p<0.01. ***p<0.001. (J-N) Side views of wild-type eyes (J) or eyes expressing Yki(K), YkiN(L), Yki△N (M) or nlsYki△N (N) with GMR-Gal4. (O-S') Wild-type third-instar larval wing discs (O-O') or wing discs expressing Yki (P-P'), YkiN (Q-Q').
Yki△N (R-R') or nlsYki△N (S-S') under the control of hhGal4 were immunostained to demonstrate the expression of Cubitus (Ci) (blue), Yki (red), Ex-lacZ (green).

**Figure 2. Importin α1 binds to N-terminus of Yki and mediates the nuclear localization of Yki**
(A) Silver staining of the sample for MS. GST and GST-YkiN protein were purified and coupled with GST beads, then pull down using S2 cell lysate. The specific bands were show by red arrowhead. (B) The results for MS. MS analysis have identified Importin α1, pen (Importin α2), Importin beta. (C) Importin α1 interacts with Yki in vitro. S2 cells were transfected with the indicated constructs. The cell lysates were immunoprecipitated, and subjected to Western blot analysis. (D) Importin α1 only interacts with YkiN. S2 cells expressing Myc-YkiN, Myc-Yki△N and Flg-Importin α1 were immunoprecipitated and probed with the indicated antibodies. (E-H) S2 cells expressing Myc-Yki (E) or Myc-Yki with HA-Importin α1 (F), HA-Importin α2 (G), HA-Importin α3 (H) were immunostained with anti-Myc (green) or anti-HA (red) antibodies. (I-J') S2 cells expressing Myc-YkiN (I-I'), Myc-Yki△N (J-J') with or without HA-Importin α1 were immunostained with anti-Myc (green) or anti-HA (red) antibodies. Nuclei are marked by DAPI (blue). (K) Cells with different nucleocytoplasmic distributions of Myc-tagged variants were counted. A total of 100 cells were counted for each case. The y axis indicates the percentage of cells in each category. (L-L') Drosophila imaginal wing discs expressing Myc-YkiN (L) or Myc-YkiN plus HA-Importin α1 (L') by MS1096 were dissected and stained with anti-Yki (green) or anti-HA (red) antibodies.

**Figure 3. In vivo, the function of Hippo signaling is dependent on Importin α1**
(A-C**') One copy of Importin α1 mutant partially blocks Yki's function. Side view (A-C), electron microscope photos (A'-C'), BrdU incorporation (A''-C'') or Diap1 expression (A'''-C'''') of wild-type eyes (A-A''') or eyes expressing Yki (B-B''') or Yki plus Importin α1+/− (C-C''') by GMR-Gal4. (D-F**') Importin α1 null clones block the function of Yki. Larval wing discs containing Importin α1 mutant (D-D'''), Yki overexpression (E-E'''), Importin α1 mutant combined with Yki (F-F''') were dissected and examined to determine the expression of Yki (D'-F') and Diap1 (D''-F''). Importin α1 mutant clones were marked by GFP signal. (G-I**') Importin α1 RNAi can partially block the function of hpo mutant. Side views of adult eyes contain Importin α1 RNAi only (G), hpo mutant only (H), hpo mutant clones plus Importin α1 RNAi (I). Drosophila imaginal eye discs expressing Importin α1 RNAi (G'-G'''), hpo mutant clones (H''-H''') or hpo mutant clones plus Importin α1 RNAi (I'-I''') using the MARCM system were dissected and stained with anti-GFP (green) or anti-Ex (red) antibodies.

**Figure 4. Mapping the important sites on Yki N-terminus**
(A) Amino acid sequence of YkiN/YkiNM/YkiNM1/YkiNM2/YkiNM3/YkiNM4/YkiNM5/YkiNM6. (B) R15A point mutation of YkiN (YkiNM2) blocks its association with Importin α1. S2 cells were transfected with the indicated plasmids and were immunoprecipitated, followed by Western blot analysis. (C) Myc-YkiN and Myc-YkiNM6 can interact with Importin α1, while Myc-YkiNM2 or Myc-YkiNM5 cannot. S2 cells were transfected with the indicated constructs. The cell lysates were immunoprecipitated and probed with anti-Myc and anti-HA antibodies. (D-I') YkiNM2 shows suppressed nuclear entrance induced by Importin α1. S2 cells were transfected with the YkiN variants and Importin α1 and then stained with anti-Myc (green) and anti-HA (red) antibodies. (J) Cells in D-I' were categorized based on anti-Myc immunostaining pattern. 100 cells were counted in each case. S2 cells expressing the indicated proteins were immunostained with the anti-Myc (green) and anti-HA (red) antibodies.

**Figure 5. Test the function of R15 in vitro and in vitro**
(A) Yki full-length point mutation (YkiM2) has no interaction with Importin α1. Co-immunoprecipitation between Myc-Yki/YkiM2 and HA-Importin α1 is performed. (B-E) YkiM2 stays in the cytoplasm when Importin α1 was co-expressed. Immunostaining of S2 cells expressing Myc-Yki (B-C) or Myc-YkiM2 (D-E) (green) with or without HA-Importin α1 (red). (F) Cells in B-E were categorized based on anti-Myc...
immunostaining pattern. 100 cells were counted in each case. (G-J’’) YkiM2 have functional defect in vivo. Drosophila imaginal eye discs expressing wide type clones (G-G’’), yki mutant clones (H-H’’), yki mutant clones plus overexpressed Yki (I-I’’) or yki mutant clones plus overexpressed YkiM2 (J-J’’) using the MARCM system were dissected and stained with anti-Diap1 (red) or anti-Yki (blue) antibodies.

Figure 6. Hippo signaling regulates Yki localization through impairing the binding between Yki and Importin α1
(A) 14-3-3 cannot interact with YkiN, but can interact with YkiΔN. (B) Hpo but not 14-3-3 affects the binding between Yki and Importin α1. S2 cells expressing the indicated constructs were immunoprecipitated and probed with the indicated antibodies. (C) The cellular localization of Myc-Yki but not that of Myc-Yki168A is influenced by Hpo. Co-immunoprecipitation experiment was performed, followed by western blot analysis. (D) The function of Hpo depends on its kinase activity. S2 cells were transfected with the indicated constructs. The cell lysates were immunoprecipitated, followed by western blot. (E-H’’) Hpo inhibits Importin α1 induced Yki localization. S2 cells were immunostained with the indicated antibodies. (I) Cells in E-H’’ were categorized based on anti-Myc immunostaining pattern. 100 cells were counted in each case. (J) The interaction between Yki and Importin α1 was Ran-GTP sensitive. S2 cells were transfected with the indicated constructs. The cell lysates were immunoprecipitated, followed by western blot.

Figure 7. The model of how Importin α1 mediates Yki nuclear import
(A) A schematic diagram of the regulation of Yki nuclear import. When Hippo signaling is on, the upstream kinase can phosphorylate Yki at its S168, make Yki stay in the cytoplasm through binding with 14-3-3 (left); while when Hippo signaling is off, Importin α1 can bind Yki through its R15, then bring Yki into nucleus to induce the target genes expression (right).
Fig1. N-terminal is essential for the nuclear localization and function of Yki
Fig 2. Importin α1 binds to Yki and mediates the nuclear localization of Yki
Fig3. In vivo, the function of Hippo signaling is dependent on Importin α1
**Fig 4. Mapping the important sites on Yki N-terminus**

**A**

YkiN: MCACLIAIILCSFRLYTISAFYMLTMSASSNTNSLIEKIDDEDMLSPIKSNN
YkiNM: MCACLIAIILCSFRLYTISAFYMLTMSASSNTNSLIEKIDDEDMLSPIKSNN
YkiNM1: MCACLIAIILCSFRLYTISAFYMLTMSASSNTNSLIEKIDDEDMLSPIKSNN
YkiNM2: MCACLIAIILCSFRLYTISAFYMLTMSASSNTNSLIEKIDDEDMLSPIKSNN
YkiNM3: MCACLIAIILCSFRLYTISAFYMLTMSASSNTNSLIEKIDDEDMLSPIKSNN
YkiNM4: MCACLIAIILCSFRLYTISAFYMLTMSASSNTNSLIEKIDDEDMLSPIKSNN
YkiNM5: MCACLIAIILCSFRLYTISAFYMLTMAANANSLIEKIDDEDMLSPIKSNN
YkiNM6: MCACLIAIILCSFRLYTISAFYMLTMAANANSLIEKIDDEDMLSPIKSNN

**B**

| IP Myc | IP HA |
|--------|-------|
| HA-a1  | +     |
| Myc-YkiN | +     |
| Myc-YkiNM | +    |
| Myc-YkiNM1 | +  |
| Myc-YkiNM2 | +  |
| Myc-YkiNM3 | +  |
| Myc-YkiNM4 | +  |

**C**

| IP Myc | IP HA |
|--------|-------|
| HA-a1  | +     |
| Myc-YkiN | +     |
| Myc-YkiNM2 | +  |
| Myc-YkiNM5 | +  |
| Myc-YkiNM6 | +  |

**D**

Myc-YkiN
Myc-YkiN
Myc-YkiNM
Myc-YkiNM
Myc-YkiNM1
Myc-YkiNM2
Myc-YkiNM3
Myc-YkiNM4

**E**

Myc-YkiN
Myc-YkiN
Myc-YkiNM
Myc-YkiNM
Myc-YkiNM1
Myc-YkiNM2
Myc-YkiNM3
Myc-YkiNM4

**F**

Myc-YkiN
Myc-YkiN
Myc-YkiNM
Myc-YkiNM
Myc-YkiNM1
Myc-YkiNM2
Myc-YkiNM3
Myc-YkiNM4

**G**

Myc-YkiN
Myc-YkiN
Myc-YkiNM
Myc-YkiNM
Myc-YkiNM1
Myc-YkiNM2
Myc-YkiNM3
Myc-YkiNM4

**H**

Myc-YkiN
Myc-YkiN
Myc-YkiNM
Myc-YkiNM
Myc-YkiNM1
Myc-YkiNM2
Myc-YkiNM3
Myc-YkiNM4

**I**

Myc-YkiN
Myc-YkiN
Myc-YkiNM
Myc-YkiNM
Myc-YkiNM1
Myc-YkiNM2
Myc-YkiNM3
Myc-YkiNM4

**J**

Percentage of cells
Fig5. Test the function of R15 in vitro and in vitro
Fig 6. Hippo signaling regulates Yki localization through impairing the binding between Yki and Importin α1.
Fig 7: The model of how Importin α1 mediates Yki nuclear import

A

Hippo signaling On

Hippo signaling Off

- Cytosol
- Nucleus
- NPC
- Diap, ban, Ex
- Yki
- Hpo
- B15
- Importin α1
- Sd
Importin α1 Mediates Yorkie Nuclear Import via N-terminal Non-canonical Nuclear Localization Signal

Shimin Wang, Yi Lu, Meng-Xin Yin, Chao Wang, Wei Wu, Jinhui Li, Wenqing Wu, Ling Ge, Lianxin Hu, Yun Zhao and Lei Zhang

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