An in vivo RNAi mini-screen in Drosophila cancer models reveals novel potential Wnt targets in liver cancer

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ABSTRACT

Background/Aims: Aberrant activation of the Wnt/β-catenin signaling, which arises from the accumulation of mutant β-catenin in the cell, is one of the most common driving forces in hepatocellular carcinoma (HCC). We previously identified several genes that are regulated on the overexpression of β-catenin in the HCC cell line that are suggested to be novel Wnt/β-catenin targets playing effective roles in cancer. The aim of the present study was to elucidate the roles of these putative target genes in tumorigenesis with an in vivo analysis in Drosophila.

Materials and Methods: We selected 15 genes downregulated in two Drosophila cancer models.

Results: The results from the RNAi mini-screen revealed novel roles for the analyzed putative Wnt/β-catenin target genes in tumorigenesis. The downregulation of the analyzed nine genes led to tumor formation as well as metastasis in Drosophila, suggesting a tumor suppressor function. On the other hand, the knockdown of the other two genes suppressed tumor and metastasis formations and disturbed the development of the analyzed eye tissues, indicating an oncogenic or developmental role for these genes.

Conclusion: These findings could serve to identify novel subjects for cancer research in order to provide insight into the diagnostic and therapeutic processes of several cancer types.

Keywords: Wnt/β-catenin signaling, RNAi, Drosophila cancer model, hepatocellular carcinoma

INTRODUCTION

Wnt signaling is an evolutionary conserved pathway in various organisms from worms to mammals and plays an important role in several biological processes, such as development, differentiation, cellular proliferation, morphology, motility, and cell fate. Wnt proteins constitute a family of secreted cysteine-rich glycoproteins that exhibit distinct expression patterns in the embryo and adult organisms (1). In mammals, 12 distinct Wnt protein families exist that might induce at least four different pathways: canonical Wnt/β-catenin/TCF, noncanonical Wnt/calcium, Wnt/planar cell polarity, and Wnt/G protein (2). However, alterations of the canonical Wnt/β-catenin/TCF pathway are implicated in tumorigenesis.

If the Wnt/β-catenin signaling pathway is not activated, cytoplasmic β-catenin levels are maintained low through continuous proteasome-mediated degradation, which is controlled by a multiprotein complex containing glycogen synthase kinase 3β (GSK-3β), adenomatous polyposis coli, and Axin.

The activation of the Wnt/β-catenin signaling pathway is initiated by binding of a Wnt ligand to Frizzled receptor and low-density lipoprotein receptor-related protein 5/6 co-receptor. In this case, Dishevelled inhibits the GSK-3β-dependent phosphorylation of β-catenin in response to the Wnt signal. Consequently, β-catenin is dissociated from the destruction complex and starts to accumulate in the cytosol. The accumulated β-catenin is then translocated into the nucleus, binds to the T cell factor (TCF)/lymphoid enhancer-binding factor family of transcription factors, and activates the expressions of several cell cycle and differentiation-related target genes, such as Axin, c-myc, and cyclin D1 (3).

Since β-catenin mutations and activated Wnt signaling pathway are found to be closely related with hepatocellular carcinoma (HCC) development and progression, we had previously performed the overexpression of mutant β-catenin in the HCC cell line human hepatoma 7 (Huh7) in order to mimic the active state of the Wnt/β-catenin signaling in HCC. Huh7 is well-differentiated and has an inactive Wnt/β-catenin pathway with no accumulation of endogenous β-catenin in the nucleus. β-Catenin levels in the cells were increased by transfection of this cell line with the constitutively active form of mutant β-catenin (S33Y), which has a missense mutation of ser-

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ine to tyrosine at codon 33 and is therefore insensitive to GSK-3β-mediated phosphorylation and proteasomal degradation. Consequently, Huh7 cells transfected with mutant β-catenin led more rapidly to larger tumors in nude mice than the cells transfected with a control plasmid (4).

In order to identify the novel transcriptional targets of the Wnt/β-catenin signaling pathway, genome-wide transcriptomic profiling analyses were performed in hyperactive β-catenin-expressing Huh7 cells by using the serial analysis of gene expression (SAGE) and microarray techniques. Finally, several putative Wnt/β-catenin target genes were detected with the differential expression profile on β-catenin induction in the HCC cell line.

More than 100 novel putative Wnt/β-catenin targets were identified using SAGE and microarray. Among them, several genes were primarily selected for further examinations according to some parameters. First, the selected genes were mostly affected by β-catenin induction (from +2 to -1) in Huh7 cells according to the results of SAGE and microarray approaches. Second, the selected genes were not associated with any specific cancer type so far. Finally, the genes were distinguished that have a homolog in *Drosophila melanogaster*. Thus, 15 putative Wnt/β-catenin target genes were selected for further experimental investigations (Supplementary Table 1). These 15 putative Wnt/β-catenin target genes are either novel genes that are not yet fully elucidated or genes that are partially defined but with no clear roles in cancer.

Mannosyl alpha-1,3-glycoprotein beta-1,2-N-acetylglucosaminyltransferase (MGAT1)

MGAT1 is a medial Golgi enzyme that catalyzes the first step in the conversion of oligomannose-type N-glycans into complex and hybrid N-glycans. Proteins on the cell surface that are N-glycosylated by MGAT1 are required for cell-cell interactions and for the binding of cytokines and other factors to the outer cell membrane. N-linked glycosylation is further important for the folding of some eukaryotic proteins (1).

Translationally controlled tumor protein 1 (TPT1)

The *TPT1* gene product has been suggested to function as an antiapoptotic protein since the overexpression of the gene inhibits apoptosis, whereas its knockdown promotes this process (6). Gene knockout studies revealed that *TPT1* deficient mice (7) and *Drosophila* (8) die early during embryogenesis, presumably due to unregulated apoptosis at a critical stage. These studies clearly indicate that *TPT1* may play a critical role in the control of cell survival in vivo.

Calmodulin 3 (CALM3)

Calmodulin is a structurally conserved and functionally preserved protein that is encoded by the *CALM3* gene. It serves as an intracellular calcium receptor and mediates the calcium regulation of cyclic nucleotide and glycogen metabolism, secretion, motility, and calcium transport (9).

TGF-β inducible nuclear protein 1 (TINP1)

TINP1 was originally identified as one of the putative tumor suppressor genes involved in the pathogenesis of human cell leukemia with an upregulated expression on the stimulation with TGF-β (10). However, in another study, the human *TINP1* gene product was identified as a nucleolar protein acting as a cell growth promoting regulator in the cell cycle progression. The overexpression of TINP1 promoted cell growth in different cell lines by regulating the G1/S transition in the cell cycle, whereas its knockdown attenuated the cell growth and dramatically blocked the cell cycle in the G1/S transition (11).

Flap structure-specific endonuclease 1 (FEN1)

The *FEN1* gene product is a structure-specific metallonuclease best known for its essential roles in the penultimate steps of Okazaki fragment maturation and long-patch base excision repair. The protein encoded by this gene removes 5′ overhanging flaps in DNA repair and processes the 5′ ends of Okazaki fragments in lagging strand during DNA synthesis. Furthermore, other studies indicate that FEN1 protein is a multifunctional nuclease that participates in distinct DNA metabolic pathways (12). Since the overexpression of this gene may confer a growth advantage to tumors, *FEN1* has been also suggested as a potential cancer therapeutic target (13).

Histidine triad nucleotide-binding protein 1 (HINT1)

HINT1 is a member of the evolutionarily conserved family of histidine triad proteins. In previous studies, mice with deletions in the *HINT1* gene are more prone to develop spontaneous hepatoma (14). Furthermore, an increased expression of *HINT1* resulted in the growth suppression of several cell lines including lung and colon cancer cell lines, suggesting a potential role for this gene in tumorigenesis as a suppressor (15).

Acidic calponin 3 (CNN3)

Calponin, which is a protein encoded by the *CNN3* gene, regulates actin cytoskeleton rearrangement, which is needed for the plasma trophoblast membranes to be-

come fusion competent (16). Furthermore, the gene is found to be upregulated in several brain tumors, suggesting a possible role for this gene in tumorigenesis (17).

Differentially expressed in FDCP 8 homolog (DEF8) (mouse)

DEF8 is a novel gene whose role in the cellular system is not yet fully elucidated. A recent molecular analysis revealed that *DEF8* is differentially expressed in primary hemopoietic tissues in mice (18).

Insulin-degrading enzyme (IDE)

Insulin-degrading enzyme (IDE) encodes a zinc metallopeptidase that degrades intracellular insulin, thereby terminating insulin activity as well as participating in intercellular peptide signaling by degrading diverse peptides, such as glucagon, amylin, bradykinin, and kallidin (19). Deficiencies in this protein's function are found to be associated with Alzheimer's disease (20) and type 2 diabetes mellitus (21).

Mortalin (heat shock 70 kDa protein 9)

Mortalin, a member of the heat shock protein 70 family, was first identified as a human mitochondrial heat shock protein, playing important roles in stress response and glucose regulation (22). Despite the role of mortalin in tumorigenesis is not fully elucidated, it is thought to exert its tumorigenic effects through various binding partners including p53 (23).

ADP-ribosylation factor 1 (ARF1)

The ARF1 protein is localized to the Golgi apparatus and has a central role in intra-Golgi transport. Furthermore, the *ARF1* expression is downregulated in human leukemia cell line depending on vitamin D treatment (24). However, the role of this gene in tumorigenesis is still largely undefined.

Cofilin 1 (CFL1)

The *CFL1* gene product is a widely distributed intracellular actin-modulating protein that binds and depolymerizes filamentous F-actin and inhibits the polymerization of monomeric G-actin in a pH-dependent manner. It is involved in the translocation of actin-cofilin complex from the cytoplasm to the nucleus (25). In a recent study, knockdown of *CFL1* in zebrafish interferes with the epibolic movement of the deep cell layer but not in the enveloping layer, and the defect can be specifically rescued by the overexpression of *CFL1*, suggesting an effective role for this gene in adhesion and cell movements (26).

Protein tyrosine phosphatase, receptor type, F (PTPRF)

The protein encoded by this gene is a member of the PTP family, which are signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation (27). PTPRF was shown to function in the regulation of epithelial cell-cell contacts at the adherens junctions (28). An increased expression level of this protein was found in the insulin-responsive tissue of obese, insulin-resistant individuals and may contribute to the pathogenesis of insulin resistance (29). However, an association between this gene and cancer has not yet been identified.

RAP1B (member of RAS oncogene family)

Rap proteins are small GTPases that belong to the Ras family. Genetic analysis of *Rap1b* function in the lower eukaryotes is critical for development, as its loss-of-function mutations are lethal in *Drosophila* (30). There is increasing evidence that Rap signaling is involved in the regulation of multiple cellular processes including cell differentiation and adhesion (31).

ARHGAP1 (Rho GTPase activating protein 1)

The *ARHGAP1* gene product is a member of the Rho GTPase family known to regulate multiple eukaryotic cell functions including actin cytoskeleton reorganization, polarity establishment, and cell growth. A recent study revealed that the ARHGAP1 protein plays an important role in regulating mammalian cell genomic stability. The *ARHGAP1* knockout primary cells show reduction of DNA damage repair ability, elevation of genomic abnormalities, and induction of multiple cell cycle inhibitors including p53, suggesting an activity for this gene in genome maintenance and cell cycle regulation (32).

MATERIALS AND METHODS

Drosophila *stocks*

ey-GAL4, GS88A8, UAS-Dl/CyO (eyeful), and ey-GAL4 fly strains were used. UAS-Dl/CyO (sensitized) flies were all kindly provided by Prof. Bassem Hassan from the University of Leuven in Belgium.[33] The following were obtained from the Vienna *Drosophila* RNAi Center: UAS-RNAi-Mgat1 (stock no.: v103609), UAS-RNAi-Tctp (stock no.: v26632), UAS-RNAi-Cam (stock no.: v102004), UAS-RNAi-Ip259 (stock no.: v110697), UAS-RNAi-Fen1 (stock no.: v108738), UAS-RNAi-HINT1 (stock no.: v110597), UAS-RNAi-Mp20 (stock no.: v40554), UAS-RNAi-DEF8 (stock

no.: v108938), UAS-RNAi-Ide (stock no.: v101317), UAS-RNAi-Hsc70-5 (stock no.: v106236), UAS-RNAi-Arf79f (stock no.: v103572), UAS-RNAi-YL-1 (stock no.: v107951), UAS-RNAi-Liprin-alpha (stock no.: v106588), UAS-RNAi-Roughened (stock no.: v9055), UAS-RNAi-RhoGAP68F (stock no.: v107775), UAS-RNAi-Axin (stock no.: v7748), and UAS-RNAi-white (stock no.: v40657) (Table 1). All flies were raised in a fly incubator at 25 °C on a standard fly food.

Examination of tumorigenesis and metastasis

Eyeful and sensitized flies with desired genes' RNAi downregulations were anesthetized with CO_2 and analyzed under a stereo microscope. For each gene's downregulation, 180 flies were analyzed in each of the eyeful and sensitized backgrounds. Tumor formations in the eye tissue and metastasis formations in the entire body were analyzed. Each eye of the flies was scored separately, and the eyes were considered with tumors when the eye tissue showed at least one folding. Metastasis formations were detected as amorphous red-pigmented cells outside of the eye field.

Statistical analysis

The results of the analyzed putative target genes' downregulations were normalized to the results of the negative control *white* gene's downregulation. Student's t-test was used to determine the significance of the differences between the obtained results. A p value <0.05 compared with the control group was considered as significantly different.

Ethics committee approval is not required because our study does not involve any human or animal tissues.

RESULTS

In vivo RNAi screening in the eyeful and sensitized Drosophila cancer models

In order to identify the possible roles of the selected 15 putative Wnt/β-catenin targets in tumorigenesis, an in vivo RNAi mini-screen has been performed in the eyeful and sensitized *Drosophila* cancer models. The *Drosophila* homologs of the selected genes were knockdowned using the RNAi system. First, the suitable transgenic UAS-RNAi fly lines of the genes of interest (Table 1) were crossed with the eyeful flies containing the ey-GAL4 constructs (Figure 1a) to enable an eye tissue-specific gene downregulation in the eyeful background. In the resulting progeny, flies with the downregulated target genes were selected, and the alterations in the existing tumor and metastasis formation prevalences on the knockdowns were examined.

Second, the candidate genes were knockdowned in the sensitized *Drosophila* cancer model in order to further investigate the potential of the same genes to induce novel tumor and/or metastasis formations. For this purpose, the transgenic RNAi fly lines were crossed with the sensitized flies bearing the ey-GAL4 drivers (Figure 1b). Thus, an eye tissue-specific knockdown of the candidate genes was achieved in the sensitized flies. In the resulting progeny, tumor and metastasis formation prevalences were examined.

In these mini-screens, three independent crosses were set up for each gene's downregulation, and from each cross, 180 flies were analyzed with the desired gene knockdown. In both of the eyeful and sensitized backgrounds, the *white* gene was used as a negative con-

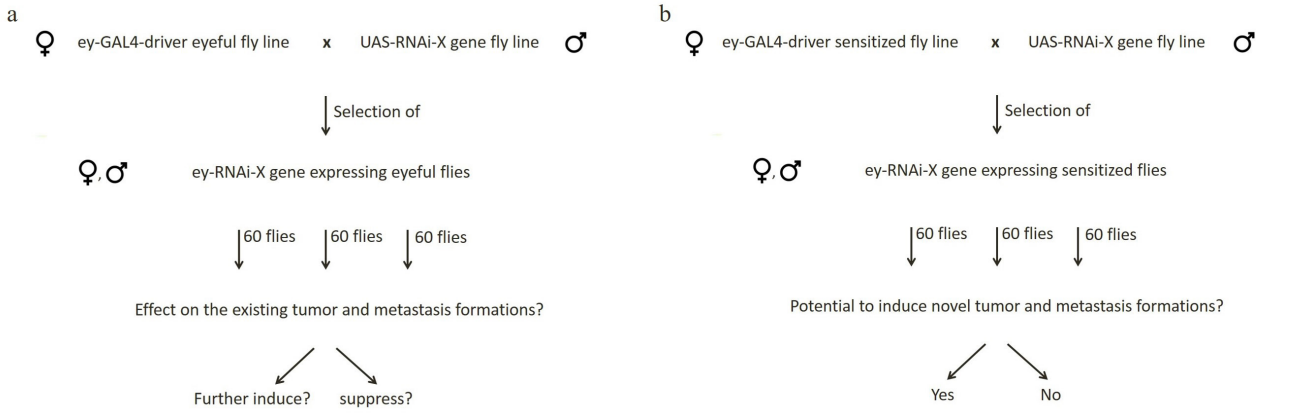

Figure 1. a, b. Experimental outline of the mini-screen in the eyeful (a) and sensitized (b) cancer models

trol that has a function in the formation of developing eye color but no effect on tumorigenesis. On the other hand, the *Axin* gene that is involved in the degradation of β-catenin as a negative regulator of the Wnt/β-catenin signaling pathway and has a tumor suppressor activity was used as a positive control.

Analysis of tumor and metastasis formation prevalences in the eyeful model

The tumor formation frequencies were examined on the downregulations of the target genes in the eyeful background. For this purpose, a total of 180 eyeful flies were analyzed for any tumor formation in the eye tissue.

Figure 2 shows several examples of the examined eyes of flies. During the analysis, each eye of the flies was scored separately. Figure 2a shows the eyeful flies with no active gene knockdown presented as a regular, single-layered, red-pigmented and round-shaped eye tissue. In comparison to eyeful flies, the downregulation of the negative control *white* gene resulted in lighter eye color formation (Figure 2b) since this gene is involved in the formation of developing eye color in *Drosophila*. When the eye tissue exhibited an excessive overgrowth and generated at least one outgrowing folding (Figure 2c, d) or additional red-pigmented eye tissue (Figure 2e, f) on the knockdowns, the eyes were accepted as tumorous. As long as the eye tissue was located on the fly head, it was considered as a tumor and not as metastasis.

The statistical analysis showed that the downregulation of the negative control *white* gene in the eyeful flies induced tumor formation in 42% of the examined eyes (data not shown here, presented as normalized data in Figure 3a). The knockdown of the positive control *Axin* gene in the eyeful flies resulted in increased frequency of eye tumors compared with the negative control *white* gene (Figure 3a). This result confirmed the known tumor

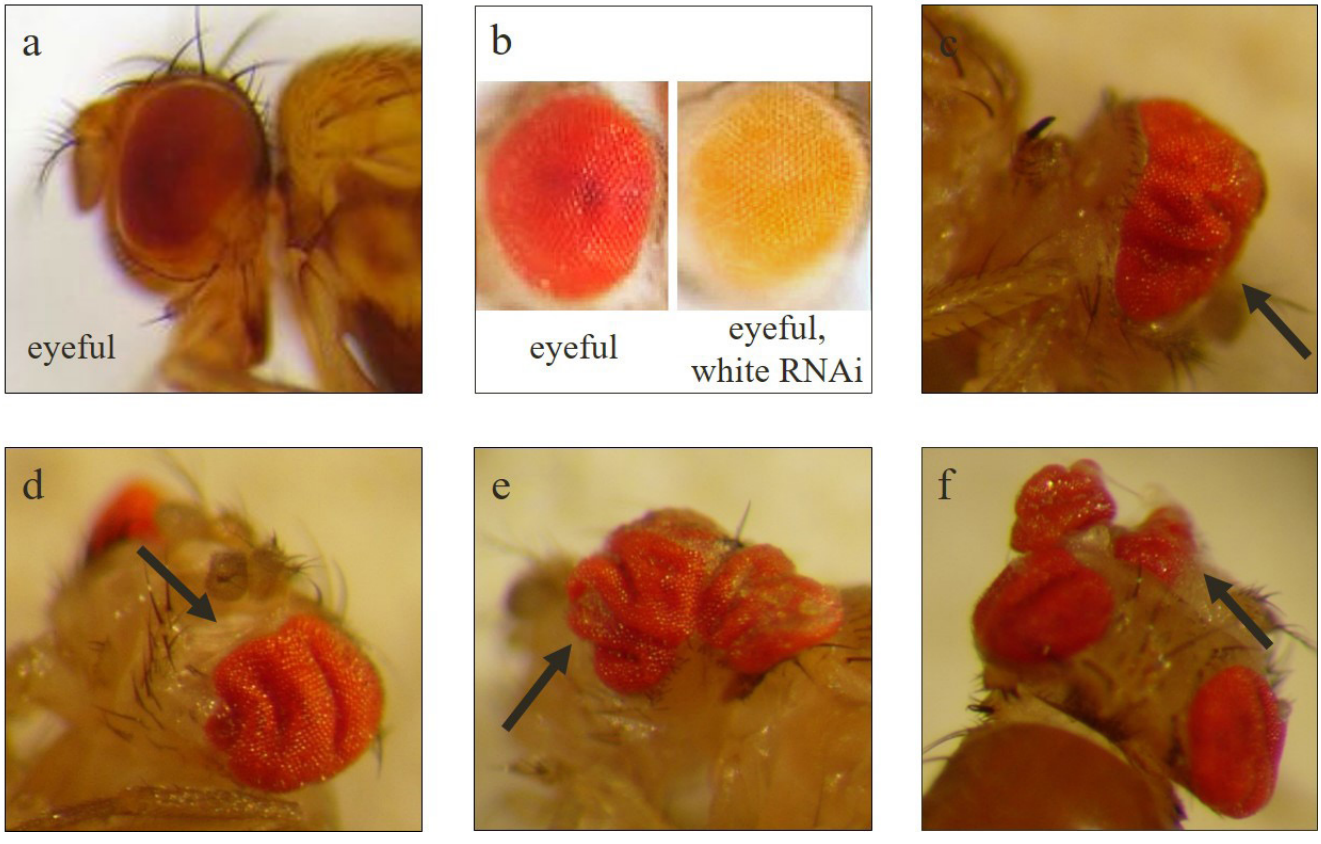

Figure 2. a-f. Several examples of tumorigenic eye tissue formations in eyeful flies. Eyeful flies present a regular red-eye phenotype (a), whereas the *white* gene knockdown leads to lighter eye color development (b), as expected. Tumor formations in flies are detected as excessive outgrowth of the eye tissue in the form of foldings (c and d) or additional eye tissue generation on the head (e and f). Arrows indicate the related examples in each picture

suppressor activity of *Axin* in the eyeful cancer model. The knockdown of 11 tested genes (*Mgat1*, *YL-1*, *HINT1*, *RhoGAP68f*, *DEF8*, *Liprin-alpha*, *Ide*, *Mp20*, *Roughened*, *Cam*, and *Tctp*) resulted in significantly increased tumor formations ranging between 1.5- and 2.3-fold (Figure 3a), indicating a potential tumor suppressor activity for each of these examined genes. On the other hand, the downregulation of the other three genes (*Hsc70-5*, *Arf79f*, and *lp259*) showed significantly decreased tumor formation prevalences (Figure 3a) in comparison to the negative control *white* gene, suggesting a putative oncogenic activity for these genes.

In addition to tumor formation prevalences, the metastasis formation frequencies have been also analyzed in the same 180 eyeful flies in a further step. Figure 3b shows the metastasis formation rates in these flies. Metastasis could be seen as amorphous red-pigmented cells outside of the eye field and formed in the dorsal part, ventral part, or neck of the flies.

The downregulation of the negative control *white* gene in the eyeful flies showed metastasis in 3% of the examined flies (data not shown here), whereas that of the positive control gene *Axin* strongly enhanced metastasis formations when downregulated (Figure 3b).

Strikingly, the knockdowns of nine genes (*Mgat1*, *YL-1*, *HINT1*, *RhoGAP68f*, *Liprin-alpha*, *Ide*, *Mp20*, *Cam*, and *Tctp*), which showed increased tumor formation prevalences, also resulted in significantly increased metastasis formation frequencies ranging between 2- and 7-fold

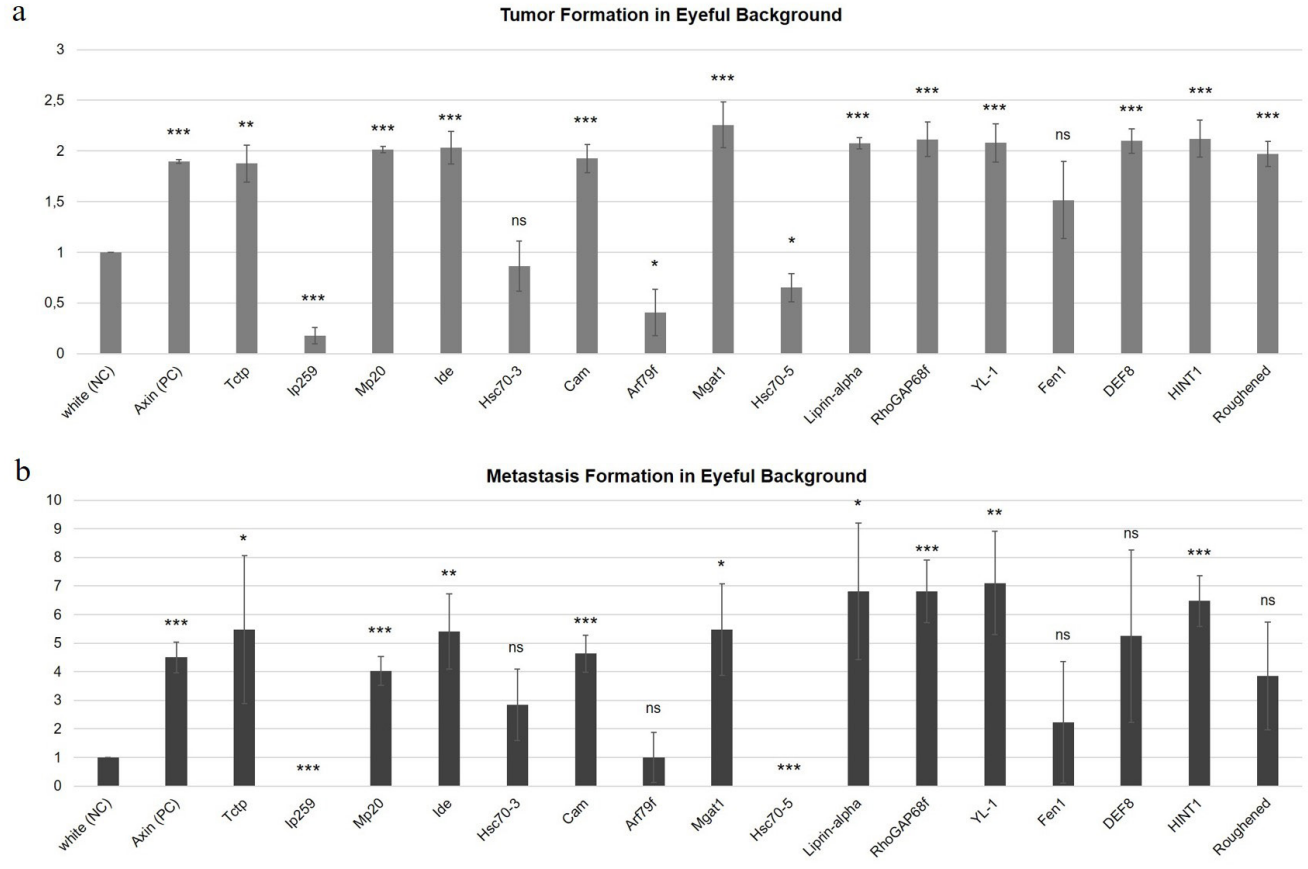

Figure 3. a, b. (a) Normalized tumor formation prevalences in the eyeful cancer model. Decreased levels of *Mgat1, YL-1, HINT1, RhoGAP68f, DEF8, Liprin-alpha, Ide, Mp20, Roughened, Cam*, and *Tctp* significantly enhance tumorigenesis. In contrast, the knockdown of *Hsc70-5* and *lp259* significantly decreases the tumor formation in the eyeful background. All results are normalized to the tumor formation rates of the downregulated negative control (NC) *white* gene. *p<0.05, **p<0.01, ***p<0.001; ns, not significant as analyzed by Student's t-test. (b) Normalized metastasis formation prevalences in the eyeful cancer model. The knockdowns of *Mgat1, YL-1, HINT1, RhoGAP68f, Liprin-alpha, Ide, Mp20, Cam,* and *Tctp* significantly increase metastasis formation frequencies in the eyeful background

(Figure 3b) in the eyeful flies, further supporting the potential tumor suppressor activity of these genes.

On the other hand, the downregulations of *Hsc70-5* and *Ip259*, which showed decreased tumor formation prevalences, were able to totally suppress the metastasis formation in the eyeful background (Figure 3b), indicating a potential oncogenic function for these two genes.

Analysis of tumor and metastasis formation prevalences in the sensitized model

Analyzing the effects of the target genes on the existing cancerous background in the eyeful flies, the next potential of the same genes to induce any tumor and/or metastasis was questioned in the sensitized model. For this purpose, 180 sensitized flies with the specific gene knockdowns were analyzed for tumorigenic and metastatic eye tissues.

Figure 4a shows the tumor formation prevalences in the analyzed sensitized flies. Knockdown of the positive control gene *Axin* strongly triggered tumor formation in the sensitized flies, confirming its tumor suppressor function. The downregulation of 11 candidate genes (*Mgat1*, *YL-1*, *HINT1*, *RhoGAP68f*, *DEF8*, *Liprin-alpha*, *Ide*, *Mp20*, *Roughened*, *Cam*, and *Tctp*), which increased tumor frequencies in the eyeful background, was able to induce tumor formation in the sensitized cancer model as well (Figure 4a). These data confirm the results obtained in the eyeful background and strikingly demonstrate a big evidence for a potential tumor suppressor function for each of these examined genes.

In contrast, the knockdown of the additional two candidate genes (*Hsc70-5* and *Ip259*), which resulted in decreased tumor and metastasis formations in the eyeful

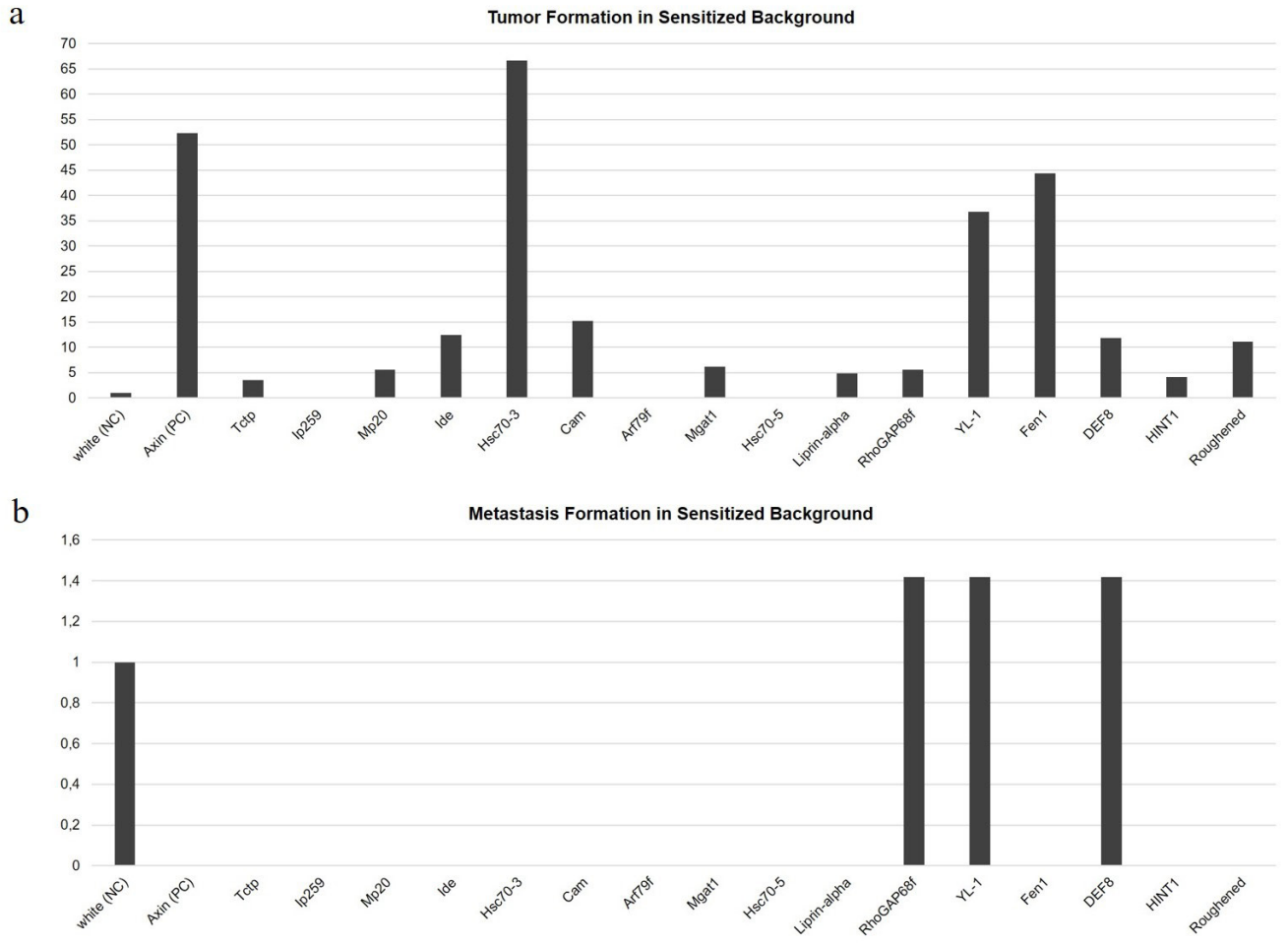

Figure 4. a, b. Normalized tumor (a) and metastasis (b) formation prevalences on RNAi downregulations in the sensitized cancer model

background, suppressed the tumor formation in the sensitized flies (Figure 4a), indicating an oncogenic function for these genes and supporting the results of the eyeful background. Furthermore, the downregulation of the same two genes resulted in very small or no-eye phenotype in 46%-58% of the fly eyes in the sensitized background (data not shown here), suggesting an effective role for each of these genes in development.

Analysis of metastasis formation prevalences in the sensitized model

After the examination of tumor formation prevalences on the downregulation of the target genes in the sensitized flies, metastasis formation frequencies were investigated in the same 180 sensitized flies in an additional step.

Figure 4b shows the metastasis formation prevalences in the analyzed sensitized flies. The positive control *Axin* gene was not able to induce metastasis formation in the sensitized background when downregulated, which could be a false-negative result due to the limited numbers of the examined flies. On the other hand, the downregulation of *YL-1* and *RhoGAP68f*, which significantly enhanced tumor and metastasis formations in the eyeful background and induced tumor formation in the sensitized model, was able to trigger metastasis in the sensitized background as well. In contrast to this, *Hsc70-5* and *Ip259* knockdowns suppressed metastasis formations in the sensitized flies consistent with the results obtained in the eyeful model (Figure 4b).

DISCUSSION

The canonical Wnt/β-catenin signaling is an evolutionary conserved pathway that is involved in various events during embryonic development, such as axis formation, cellular proliferation, differentiation, and morphogenesis. In addition to its role in development, the Wnt/β-catenin pathway has the potential to initiate tumor formation when it is aberrantly activated. Molecular studies have revealed that activating mutations in the Wnt/β-catenin signaling pathway are responsible for approximately 90% of colorectal cancer and somewhat less frequently in other cancer types, such as HCC. Those characteristics of the Wnt/β-catenin signaling pathway make the pathway itself and its targets important key subjects for cancer studies.

In order to identify novel transcriptional targets of the Wnt/β-catenin pathway, a microarray and SAGE screen was conducted in our laboratory. Consequently, a number of genes were differentially regulated on the accumulation of mutant β-catenin in the human HCC cell line Huh7, by means of mimicking the active state of the Wnt/β-catenin pathway. These genes are considered as novel candidates of the canonical Wnt/β-catenin signaling pathway.

Since β-catenin mutations and an activated Wnt signaling pathway have been found to be closely related with tumorigenesis, the significantly and differentially regulated putative target genes of the canonical Wnt/β-catenin signaling pathway may play effective roles in cancer. The aim of the present study was to identify the possible effects of the selected 15 candidate genes on tumor and metastasis formations and characterize their potential roles in cancer. For this purpose, an in vivo RNAi mini-screen was performed in two *Drosophila* eye cancer models: eyeful and sensitized. In these cancer models, the *Drosophila* homologs of the selected 15 genes were perturbed, and the effects of the altered gene expressions on tumor formation and metastasis were examined.

In the eyeful cancer model, the concurrent overexpression of the Notch ligand Delta and two epigenetic silencers lola and pipsqueak resulted in tumor formation and metastasis in flies. When knockdowned in this background, the nine analyzed putative Wnt/β-catenin target genes (*Mgat1*, *YL-1*, *HINT1*, *RhoGAP68f*, *Liprin-alpha*, *Ide*, *Mp20*, *Cam*, and *Tctp*) further enhanced the tumor and metastasis rates. In contrast to this, *Hsc70-5* and *Ip259* knockdowns totally suppressed the existing tumor and metastasis and resulted in smaller or no-eye phenotypes.

Further, the effects of the downregulations of the same genes were examined in the sensitized cancer model by ignoring the overexpression of lola and pipsqueak from the tumor-inducing background of eyeful flies. In this background, further supporting the results in the eyeful background, the knockdowns of nine candidate genes (*Mgat1*, *YL-1*, *HINT1*, *RhoGAP68f*, *Liprin-alpha*, *Ide*, *Mp20*, *Cam*, and *Tctp*) induced tumorigenesis, whereas those of two of them (*YL-1* and *RhoGAP68f*) were also able to promote metastasis formation when downregulated. On the other hand, decreased levels of *Hsc70-5* and *Ip259* did not trigger any tumor or metastasis formation and resulted again in smaller or no-eye phenotypes, providing clues about a possible role for these genes in developmental pathways and linking differentiation and tumorigenesis in *Drosophila*. Overall, the results obtained from the eyeful and sensitized backgrounds are consistent with each other and suggest a tumor suppressor function for the analyzed nine candidate genes and an oncogenic or a developmental function for the other two genes for the first time.

However, the exact functions of the examined genes in cell proliferation and apoptosis mechanisms in the cells are still unclear. The examination of the effects of these genes on cell proliferation and apoptosis would be interesting by analyzing the levels of proliferation markers or apoptosis markers in the eye tissues of eyeful, sensitized, and wild-type flies on the downregulation of these candidate genes. These data may provide clues about the potential roles of these genes in tumorigenesis by identifying their effects on cell proliferation or apoptosis. Furthermore, these genes were identified as novel potential Wnt/β-catenin target genes since they were found to be differentially expressed on β-catenin induction, mimicking overactivated Wnt signaling. Therefore, in addition to their potential tumorigenic functions, the identification of their interaction partners and putative roles in the Wnt/β-catenin signaling pathway is crucial in order to enlighten the molecular mechanisms and signaling networks in which they are involved in. Thus, a possible cross-talk between the Wnt/β-catenin signaling pathway and other signaling cascades via these putative target genes might also be elucidated eventually.

In the present study, we identified novel potential Wnt/β-catenin target genes with possible tumor suppressor or oncogenic functions that may be considered as novel subjects for future cancer studies. By elucidating the possible roles of these genes in tumorigenesis and metastasis formations and clarifying the molecular mechanisms behind their activities, these genes may be identified as novel targets for diagnostic and therapeutic processes of liver cancer as well as several other cancer types.

Ethics Committee Approval: Ethics committee approval is not required because our study does not involve any human or animal tissues.

Informed Consent: N/A.

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Conflict of Interest: The authors have no conflict of interest to declare.

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Supplementary Table 1. Human and the corresponding *Drosophila* gene names

Human	*Drosophila*
MGAT1	*Mgat1*
TPT1	*Tctp*
CALM3	*Cam*
TINP1	*Ip259*
FEN1	*Fen1*
HINT1	*HINT1*
CNN3	*Hsc70-3*
DEF8	*DEF8*
IDE	*Ide*
Mortalin	*Hsc70-5*
ARF1	*Arf79f*
CFL1	*YL-1*
PTPRF	*Liprin-alpha*
RAP1B	*Roughened*
ARHGAP1	*RhoGAP68f*