IDENTIFICATION AND *IN VIVO* ANALYSIS OF PUTATIVE WNT TARGET GENES WITH EFFECTIVE ROLES IN CANCER

by

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To my beloved family...

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ABSTRACT

IDENTIFICATİON AND *IN VIVO* ANALYSIS OF PUTATIVE WNT TARGET GENES WITH EFFECTIVE ROLES IN CANCER

One of the most common driving forces in hepatocellular carcinoma (HCC) is the aberrant activation of the Wnt/β-catenin signaling due to the accumulation of mutant β-catenin in the cell. Our group previously detected several genes with altered expression levels upon the overexpression of β-catenin in the HCC cell line, which are suggested to be novel Wnt/β-catenin targets that may play effective roles in cancer. In this study, our aim was to elucidate the roles of these putative Wnt/β-catenin target genes in tumorigenesis with an *in vivo* analysis in *Drosophila*. For this purpose, we downregulated the selected 16 putative Wnt/β-catenin target genes in two *Drosophila* cancer models using RNA interference (RNAi) and examined their effects on tumor and metastasis formations. Results from the RNAi screen revealed novel roles for the analyzed 16 putative Wnt/β-catenin target genes in tumorigenesis. The analyzed 13 putative Wnt/β-catenin target genes' downregulations promoted tumor and metastasis formations in *Drosophila*, suggesting a tumor suppressor function; whereas the other 3 genes' downregulations suppressed the tumor and metastasis formations and hindered the development in the analyzed tissues, suggesting an oncogenic or developmental role for these genes. These findings could serve to identify novel subjects for cancer research in order to provide insight into diagnostic and therapeutic processes of several cancer types as well as further characterization of the canonical Wnt/β-catenin signaling pathway.

ÖZET

KANSERDE ETKİN ROL OYNAYAN OLASI WNT HEDEF GENLERİNİN BELİRLENMESİ VE *İN VİVO* ANALİZİ

Hepatoselüler karsinoma (HCC) gelişiminde rol oynayan tetikleyici faktörlerden biri de, hücre içinde mutant β-katenin birikimi sonucu ortaya çıkan Wnt/β-katenin sinyal yolağının olağandışı aktivasyonudur. Daha önce grubumuz tarafından, HCC hücre hattında anlatımları β-katenin'in yüksek anlatımına bağlı olarak değişen çeşitli genler tespit edilmiştir. Bu genlerin, Wnt/β-katenin sinyal yolağında yer alan olası hedef genler olarak görev yaptığı ve kanserde etkin rol oynayabileceği düşünülmektedir. Bu çalışmada, tespit edilen olası Wnt/β-katenin hedef genlerinin tümör oluşumundaki rollerinin *Drosophila*'da *in vivo* bir analiz ile aydınlatılması amaçlanmıştır. Bu amaçla, seçilen 16 olası Wnt/β-katenin hedef genin anlatımları iki farklı *Drosophila* kanser modelinde RNA interferens (RNAi) tekniği ile baskılanmış, bu baskılamaların tümör ve metastaz oluşumlarına olan etkileri incelenmiştir. Gerçekleştirilen bu RNAi analizinin sonuçları, incelenen 16 olası hedef gen için tümör oluşumunda yeni roller ortaya koymuştur. Analiz edilen 13 olası hedef genin baskılanması, tümör ve metastaz oluşumlarını tetikleyerek bu genler için tümör baskılayıcı yönde bir rol ortaya koymuştur. Diğer 3 hedef genin baskılanması ise tümör ve metastaz oluşumlarını azaltarak ve analiz edilen dokularda gelişimi olumsuz yönde etkileyerek, bu genler için onkogenik ya da gelişimsel bir rol ortaya koymuştur. Elde edilen bu bulgular, Wnt/β-katenin sinyal yolağının karakterizasyonuna destek vererek çeşitli kanser türlerinde diagnostik ve terapötik süreçlere ışık tutacak ve kanser araştırmalarına katkı sağlayacaktır.

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LIST OF SYMBOLS

µg	Microgram
µl	Microliter
µM	Micromolar
kDa	Kilodalton
mg	Miligram
min	Minutes
ml	Milliliter
mM	Millimolar
mm	Millimeter
sec	Seconds

LIST OF ACRONYMS/ABBREVIATIONS

APC	Adenomatous polyposis coli
ARF1	ADP-ribosylation factor 1
Ago2	Argonaute2
ARHGAP1	Rho GTPase activating protein 1
bp	Base pair
CALM3	Calmodulin 3
CamKII	Calmodulin-dependent kinase II
cDNA	Complementary deoxyribonucleic acid
CFL1	Cofilin 1
CKIα	Casein kinase Iα
CNN3	Acidic calponin 3
CO_2	Carbon dioxide
Cyo	Curled wing marker
Dcr-2	Dicer
ddH_2O	Double-distilled water
DEF8	Differentially expressed in FDCP 8 homolog (mouse)
Dl	Delta
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleosidetriphosphate
Dsh	Dishevelled

dsRNA	Double-stranded ribonucleic acid
EDTA	Ethylenediaminetetraacetate
ey	Eyeless
FEN1	Flap structure-specific endonuclease 1
Fz	Frizzled
GSK3β	Glycogen synthase kinase 3β
GTP-BP	GTP-binding protein
hpRNA	Hairpin ribonucleic acid
HCC	Hepatocellular carcinoma
HEK293FT	Human embryonic kidney fibroblast
HINT1	Histidine triad nucleotide binding protein 1
Huh	Human hepatoma
IDE	Insulin-degrading enzyme
Kny	Knypek
LEF	Lymphoid enhancer factor
LRP	Low density lipoprotein receptor-related protein
MENA	Enabled Homolog
MGAT1	Mannosyl alpha-1,3-glycoprotein beta-1,2-N-acetyl glucosaminyl-transferase
$MgCl_2$	Magnesium chloride
Mortalin	Heat shock 70 kDa protein 9
mRNA	Messenger ribonucleic acid
PCP	Planar cell polarity
PCR	Polymerase chain reaction

PDE	Phosphodiesterase
PKC	Protein kinase C
PLC	Phospholipase C
PTP	Protein tyrosine phosphatase
PTPRF	Protein tyrosine phosphatase, receptor type, F
RAP1B	Member of RAS oncogene family
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RT-PCR	Reverse-transcriptase mediated polymerase chain reaction
SAGE	Serial analysis of gene expression
siRNA	Small interfering ribonucleic acid
TAE	Tris-acetic acid EDTA
TCF	T-cell factor
TINP1	TGF-β inducible nuclear protein I
TPT1	Translationally controlled tumor protein 1
UAS	Upstream activating sequences
VDRC	Vienna *Drosophila* RNAi center

1. INTRODUCTION

1.1. Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is one of the primary hepatic neoplasms which arise from hepatocytes, the major cell type of the liver. It is the most common type of malignant primary liver cancer, representing 75-90% of all cases in most countries (Wang *et al.*, 2002). In developing countries the incidence rates are two to three fold higher than in developed countries. The disease is more prevalent in parts of Africa and Asia than in continental America and Europe with a strong etiological association with viral hepatitis, known hepatic carcinogens and toxins such as aflatoxin. Almost 80% of cases are due to underlying chronic hepatitis B and C virus infections. Alternatively, aflatoxin, which is a toxic carcinogen from certain *Aspergillus* species of fungus, may lead to the development of HCC by inducing acute hepatic necrosis and resulting later in cirrhosis, that may trigger the carcinogenesis of hepatocytes (Tanaka *et al.*, 2011).

With approximately 1.000.000 new cases per year, HCC is among the most prevalent and lethal cancers in the human population. However, there is only an elemental understanding of the molecular, cellular and environmental mechanisms that drive the disease pathogenesis and despite major efforts to improve diagnosis and treatment, therapeutic options remain limited (Meier *et al.*, 2009).

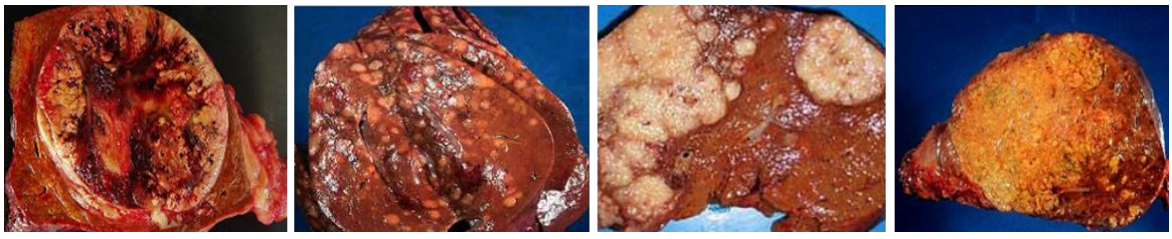

Figure 1.1: Human livers with HCC (Meier *et al.*, 2009)

The development and progression of HCC in a chronically diseased liver, frequently referred to as hepatocarcinogenesis, is a multistep and longterm process characterized by the aberrant growth and malignant transformation of liver parenchymal cells, followed by

vascular invasion and metastasis (Teoh, 2009) (Figure 1.1). Molecular mechanisms behind this process have not been completely elucidated yet. However, it is hypothesized that like most solid tumors, the development and progression of HCC may be caused by the accumulation of genetic changes resulting in altered expression levels of cancer-related genes, such as oncogenes or tumor suppressor genes or genes involved in different regulatory pathways (Zhang *et al.*, 2005).

Studies on HCC molecular profiling have revealed many HCC-associated deregulated genes and signaling pathways in which Wnt/β-catenin signaling has been proposed to be critical. The detection of β-catenin mutation in HCC several years ago indicated the significant role of the Wnt/β-catenin signaling pathway in hepatocellular carcinogenesis and tumor differentiation which has provided an essential step towards the understanding of the genesis of liver cancers (Ji *et al.*, 2011).

1.2. The Wnt Signaling Pathway

The Wnt signaling pathway is an evolutionary-conserved pathway in various organisms from worms to mammals which plays important roles 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 embryo and adult organisms (Cadigan *et al.*, 1997). In mammals, 12 distinct Wnt protein families exist which might induce at least 4 different pathways: The canonical Wnt/β-catenin/TCF pathway, and the noncanonical Wnt/calcium, Wnt/planar cell polarity (PCP) and Wnt/G protein pathways (Wodarz *et al.*, 1998).

1.2.1. Noncanonical Wnt Signaling Pathways

The most distinctive differences between the canonical Wnt/β-catenin pathway and the non-canonical pathways include the specific ligands activating each pathway. Ligands that activate the non-canonical pathways are Wnt4, Wnt5a, and Wnt11. In the Wnt/calcium pathway, Wnt ligands bind to Frizzled (Fz) and co-receptor Knypek (Kny) or Ror2. This

binding triggers the activation of calcium/calmodulin-dependent kinase II (CamKII) and protein kinase C (PKC) which will lead to multiple further changes in the cell. In the Wnt/PCP pathway, Dishevelled (Dsh) activation leads to the activation of some GTPases such as Rho and Cdc42.

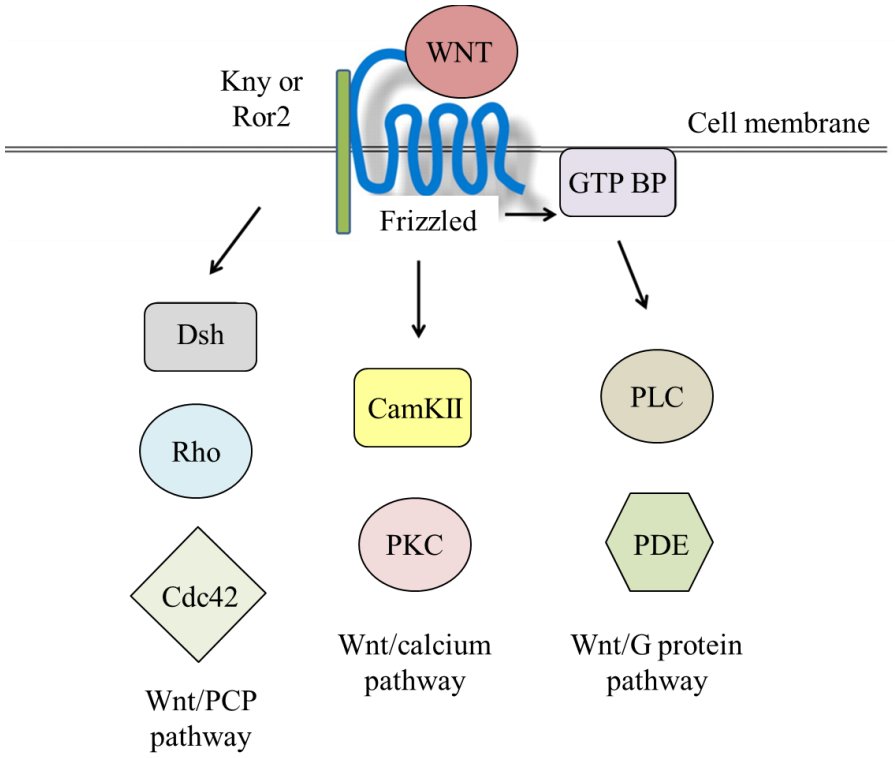

Figure 1.2. Noncanonical Wnt signaling pathways (adapted from Kohn *et al*, 2005).

Alternatively; there is also a G protein linked pathway acting downstream of Wnt/Fz binding. Concordant with the fact that Fz is a seven trans-membrane domain containing receptor which has some homology with G protein coupled receptors, Wnt binding might lead to the activation of phospholipase C (PLC) and phosphodiesterase (PDE), via G proteins (Figure 1.2.). Although the Wnt signaling contributes to multiple developmental events during embryogenesis and in homeostasis of adult tissues, the roles of noncanonical Wnt pathways are poorly understood and further investigations are needed to elucidate their effects in the cellular network (Kohn *et al.*, 2005).

1.2.2. The Canonical Wnt/β-catenin Signaling Pathway

The canonical Wnt/β-catenin signaling pathway is the best understood Wnt signaling pathway. An essential component and the key regulator of this signaling is the β-catenin molecule. It is localized with the membrane bound E-cadherin or is free in the cytoplasm, and plays a major role in the transduction of the Wnt/β-catenin signal (Takeichi *et al.*, 1991).

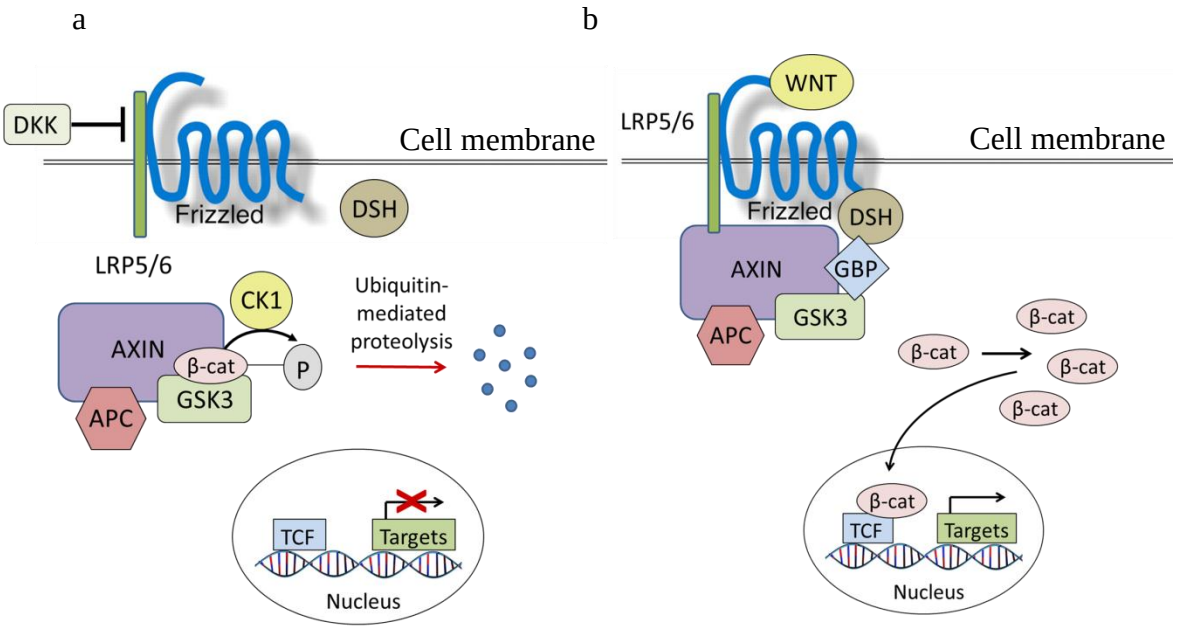

Figure 1.3. Wnt/β-catenin signaling cascade in its "inactive" (a) and "active" (b) states (Barker *et al.*, 2006).

The canonical Wnt/β-catenin signaling pathway is summarized in Figure 1.3. Cytoplasmic β-catenin levels are normally kept low through continuous proteasome mediated degradation which is controlled by a multiprotein complex containing Glycogen Synthase Kinase 3β (GSK-3β), Adenomatous Polyposis Coli (APC) and Axin. In the absence of a Wnt signal, β-catenin is present in the Axin complex. In this complex, cytosolic β-catenin, but not the cadherin-bound β-catenin, is continuously phosphorylated, ubiquitinated and degraded by proteasome. The multiprotein complex that is responsible for β-catenin degradation is assembled around the scaffold protein axin. Within this destruction complex, β-catenin is phosphorylated first by Casein Kinase Iα (CKIα) at

serine 45 and then by GSK-3β on its serine/threonine residues 33, 37 and 41. Subsequently, β-catenin is targeted for degradation via the ubiquitin-proteasome pathway (Figure 1.3a), after it is ubiquitinated by β-transduction repeat containing protein which is an E3 ubiquitin ligase (Rubinfeld *et al*., 1996).

The activation of the Wnt/β-catenin signaling pathway is initiated by binding of a Wnt ligand to Fz and Low density lipoprotein receptor-related protein (LRP) 5/6 co-receptor. In this case, Dsh 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 (Figure 1.3b), binds to the T-cell factor/Lymphoid Enhancer Factor (TCF/LEF) 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* (Behrens *et al*., 1996).

1.2.3. Role of the Wnt/β-catenin Signaling Pathway in Cancer

Aberrant activation of the Wnt/β-catenin signaling pathway can result in a wide range of pathological phenotypes or predisposition to diseases. Mutations that cause constitutive activation of the Wnt/β-catenin signaling pathway lead to different types of cancer. Several molecules in this pathway are found to be associated with different carcinomas. Among them, especially ß-catenin is found to be mutated and its accumulation is frequently observed in many cancer types (Polakis, 2007).

Mutant β-catenin molecules that are resistant to downregulation by GSK-3β phosphorylation and ubiquitination have been characterized in a variety of carcinomas including ovarian, skin and intestinal cancers (Sadot *et al.*, 2001). In colon cancers, immunohistochemical studies have demonstrated increased expression of β-catenin and its nuclear localization in tumours harboring either APC defects or β-catenin mutations in the GSK-3β phosphorylation domain (Morin *et al.*, 1997). Nuclear and cytoplasmic localization of β-catenin have been frequently seen in ovarian, uterine carcinomas and

melanomas (Morin, 1999). In addition to these, in human HCC, mutations of the β-catenin gene have been reported in 19 to 41% of primary tumours (Levy *et al.*, 2006).

The dysregulation of β-catenin has been established in several tumors. These dysregulations leading to β-catenin hyperactivation mimic active Wnt/β-catenin pathway that might result in aberrant activation of cell cycle mechanisms, which is a major mark of tumor initiation (Behrens *et al.*, 1996). Aberrant activation of the Wnt/β-catenin signaling pathway triggered by β-catenin mutations contributes significantly to the genesis of HCC and many other cancer types (Wong *et al.*, 2001). Therefore, identification of novel transcriptional targets and further characterization of the Wnt/β-catenin signaling pathway is crucial in order to provide insight into diagnostic and therapeutic processes of liver cancers as well as further general knowledge about the canonical Wnt pathway.

1.2.4. Identification of Novel Wnt/β-catenin Target Genes

Since β-catenin mutations and activated Wnt signaling pathway are found to be closely related with HCC development and progression, our research group had previously performed 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. The human HCC cell line Huh7 is well-differentiated and has an inactive Wnt/β-catenin pathway with no accumulation of endogenous β-catenin in the nucleus. By transfection of this cell line with the constitutively active form of mutant β-catenin (S33Y), which has a missense mutation of tyrosine for serine at codon 33 and is therefore insensitive to GSK-3β–mediated phosphorylation and proteasomal degradation, β-catenin levels in the cells were increased. Consequently, Huh7 cells transfected with mutant β-catenin led more rapidly to larger tumors in nude mice in comparison to cells transfected with a control plasmid (Kavak *et al.*, 2010).

In order to identify 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 SAGE (Serial analysis of gene expression) and

microarray techniques. Eventually, several putative Wnt/β-catenin target genes were detected with differential expression profile upon β-catenin induction in the HCC cell line.

Using SAGE and microarray, more than 100 novel putative Wnt/β-catenin target genes were detected, and among them, several genes were primarily selected for further examinations according to some parameters: First, the genes were selected which were mostly affected by β-catenin induction (from +2 up to -1) in Huh7 cells according to the results of SAGE and microarray approaches. Second, the genes were chosen which are not known to be associated with any specific cancer type according to the literature. Third, the genes were distinguished which have a homolog in *Drosophila melanogaster* and by this way, 16 putative Wnt/β-catenin target genes were selected for further experimental investigations.

1.2.5. Selected 16 Putative Wnt/β-catenin Target Genes

The 16 putative Wnt/β-catenin target genes which were determined for further analyses are either novel genes which are not fully elucidated yet or genes which are defined in limited levels, but their roles in cancer are still largely undefined. These selected 16 candidate genes are presented in the Table 1.1.

Table 1.1. Selected putative Wnt/β-catenin target genes with differential expression profile upon β-catenin induction.

	Human gene	***Drosophila* homolog**	**Expression levels upon β-catenin induction**
1.	*MGAT1*	*Mgat1*	downregulated
2.	*TPT1*	*Tctp*	downregulated
3.	*CALM3*	*Cam*	downregulated
4.	*TINP1*	*Ip259*	upregulated
5.	*MENA*	*Ena*	upregulated
6.	*FEN1*	*Fen1*	downregulated
7.	*HINT1*	*CG2862*	downregulated
8.	*CNN3*	*Mp20*	downregulated
9.	*DEF8*	*CG11534*	downregulated
10.	*IDE*	*Ide*	downregulated
11.	*Mortalin*	*Hsc70-5*	upregulated
12.	*ARF1*	*Arf79f*	upregulated
13.	*CFL1*	*YL-1*	downregulated
14.	*PTPRF*	*Liprin-alpha*	downregulated
15.	*RAP1B*	*Roughened*	downregulated
16.	*ARHGAP1*	*RhoGAP68F*	upregulated

1.2.5.1. *MGAT1 (Mannosyl alpha-1,3-glycoprotein beta-1,2-N-acetyl glucosaminyl transferase).* *MGAT1* encodes a transmembrane protein located in the medial compartment of the golgi apparatus with its catalytic domain within the lumen. It catalyzes the first step in the conversion of oligomannose to N-glycans of glycoproteins. 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 (Yen *et al.*, 2002).

Post-translational modifications such as glycosylation can affect the function of a single gene and mutations in the glycosylation proteins may therefore have great effects on the development of normal cells. Null mutation experiments of the mouse *MGAT1* gene suggest a fundamental role in normal development for this gene (Shi *et al.*, 2004), and mutation or dysregulation of several enzymes dependent on *MGAT1* action are found to be associated with human diseases such as carbohydrate-deficient glycoprotein syndromes (Jaeken *et al.*, 1994).

To examine the roles of the N-glycans in development and diseases, another member of the *MGAT* gene family, the *MGAT5* gene was analyzed. Like *MGAT1*, *MGAT5* is also required in the biosynthesis of N-linked glycans (Park *et al.*, 1999). However, it has been shown that mice deficient in *MGAT5* resulted in suppression of induced tumor growth and metastasis proposing an oncogenic activity for *MGAT5* (Granovsky *et al.*, 2000). These data suggest a putative link between the N-linked glycans and cancer progression and also possible roles of *MGAT* gene family members in cancer.

1.2.5.2. *TPT1 (Translationally Controlled Tumor Protein 1).* *TPT1* gene product has been suggested to function as an antiapoptotic protein since overexpression of the gene inhibits apoptosis whereas knockdown of it promotes this process (Diraison *et al.*, 2010). Gene knockout studies revealed that *TPT1* deficient mice (Chen *et al.*, 2007) and *Drosophila* (Hsu *et al.*, 2007) die early during embryogenesis, presumably due to unregulated apoptosis at a critical stage. It has been shown that *TPT1* antagonises apoptosis by enhancing the anti-apoptotic actions of MCL-1 and BCL-XL, and by anchoring into the

mitochondrial membrane in a way which inhibits dimerisation of the proapoptotic protein BCL2- associated X protein (Susini *et al.,* 2008). These studies clearly indicate that *TPT1* may play a critical role in the control of cell survival *in vivo*.

Apart from its anti-apoptotic function, *TPT1* is found to be involved in the regulation of cell growth and proliferation, since the tissue-specific knockdown of *Tctp* (the *Drosophila* homolog of *TPT1*) in *Drosophila* resulted in smaller eye and wing sizes. Furthermore, this gene in *Drosophila* was shown to control the cell growth and proliferation by regulating GTPase activity of a Ras homologue, Rheb (Hsu *et al.,* 2007).

1.2.5.3. *CALM3 (Calmodulin 3).* Calmodulin is a structurally conserved and functionally preserved protein which 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 (Zhang *et al.,* 1999). Calmodulin is also a dynamic component of the mitotic apparatus. Together with CEP110 and centrin, it is involved in a genetic pathway that regulates the centrosome cycle and progression through cytokinesis (Gusev *et al.,* 2002).

1.2.5.4. *TINP1 (TGF-β Inducible Nuclear Protein I).* *TINP1* was originally identified as one of the putative tumor suppressor genes involved in the pathogenesis of human cell leukemia with an upregulated expression upon the stimulation with TGF-β (Wu *et al.,* 1999). On the other hand, in a recent study human *TINP1* gene product was identified as a nucleolar protein acting as a cell growth promoting regulator in the cell cycle progression. Its overexpression 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 G1/S transition (Zhang *et al.,* 2010).

1.2.5.5. *MENA (Enabled Homolog).* Many actin-associated proteins play important roles in carcinogenesis of various types of cancers. MENA is also an actin-regulatory protein which belongs to the ENA/VASP protein family. Members of this protein family are

localized at the tips of protruding lamellipodia and filopodia and adhesion foci; and they are involved in the control of cell motility and cell-cell adhesion which are important subjects for development of metastatic potential (Krause *et al.*, 2003).

Recently, it has been suggested that *MENA* may have a role in human carcinogenesis. In ~75% of primary breast cancers, this gene is reported to be overexpressed, and its overexpression correlates to the poor prognostic phenotype, suggesting a potential role for the gene in tumorigenesis (Di Modugno *et al.*, 2004).

1.2.5.6. *FEN1 (Flap Structure-Specific Endonuclease 1).* *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 DNA synthesis. Furthermore, FEN1 protein has also been implicated in other major DNA metabolic pathways, including resolution of tri-nucleotide repeat sequence-derived secondary structures, rescue of stalled DNA replication forks, maintenance of telomere stability and apoptotic fragmentation of DNA. Compelling evidence from *in vitro* and *in vivo* studies indicates that FEN1 protein is a multifunctional nuclease that participates in distinct DNA metabolic pathways (Liu *et al.*, 2004).

Interestingly, *FEN1* has been suggested to promote cancer in two very different ways: (i) mutation of the gene can result in genomic instability and initiate malignant transformation and (ii) overexpression of the gene may confer a growth advantage to tumors. In response to the latter way to promote cancer, *FEN1* has been also suggested as a potential cancer therapeutic target (Zheng *et al.*, 2010).

1.2.5.7. *HINT1 (Histidine Triad Nucleotide Binding Protein 1).* *HINT1* is a member of the evolutionarily conserved family of histidine triad proteins. In previous studies, it has been revealed that mice with deletions in the *HINT1* gene are more prone to develop spontaneous hepatoma (Li *et al.*, 2006). Furthermore, 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 (Wang *et al.*, 2006).

1.2.5.8. *CNN3 (Acidic Calponin 3).* Three *CNN* gene family proteins are characterized by the N-terminal calponin homology domain and a middle region containing actin binding site-1 and -2, and are distinguished from each other by their unique C-terminal tails: basic, neutral, and acidic *CNN*s, or *CNN1*, *CNN2*, and *CNN3*, respectively. Calponin, a protein encoded by the gene *CNN3*, was originally identified as a molecule binding to F-actin, calmodulin, and tropomyosin and regulating the contraction/relaxation cycle in smooth muscle cells (Shibukawa *et al.*, 2010). Furthermore, the gene is found to be upregulated in several brain tumors suggesting a possible role for this gene in tumorigenesis (Najafov *et al.*, 2012).

1.2.5.9. *DEF8 (Differentially Expressed In FDCP 8 Homolog (Mouse)).* *DEF8* is a novel gene whose role in cellular system is not fully elucidated yet. A recent molecular analysis revealed that *DEF8* is differentially expressed in primary haemopoietic tissues in mice (Ronquist *et al.*, 2011). However, several other studies are necessary to identify the function of this gene in cellular mechanism.

1.2.5.10. *IDE (Insulin-Degrading Enzyme).* This gene encodes a zinc metallopeptidase which degrades intracellular insulin, and thereby terminates insulin activity, as well as participating in intercellular peptide signalling by degrading diverse peptides such as glucagon, amylin, bradykinin, and kallidin (Authier *et al.*, 1995). The preferential affinity of this enzyme for insulin results in insulin-mediated inhibition of the degradation of other peptides such as beta-amyloid. Deficiencies in this protein's function are found to be associated with Alzheimer's disease (Kurochkin *et al.*, 1994) and type 2 diabetes mellitus (Duckworth *et al.*, 1998). Besides insulin and beta-amyloid, several other physiologically active peptides have also been identified as high-affinity substrates for IDE *in vitro*, such as insulin-like growth factor II, tumor growth factor-α, and atrial natriuretic peptide (Shen *et al.*, 2006).

1.2.5.11. *Mortalin (Heat Shock 70kDa Protein 9).* *Mortalin*, a member of the heat shock protein 70 family, was first identified as human mitochondrial heat shock protein, having important roles in stress response and glucose regulation (Qu *et al.*, 2011). 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. Functional inactivation of tumor suppressor p53 is a common event in early carcinogenesis, and mortalin has been shown precisely in colocalization with wild-type p53, suggesting a putative role for this gene in tumorigenesis (Lu *et al.*, 2011).

1.2.5.12. *ARF1 (ADP-Ribosylation Factor 1).* *ARF1* is a member of the human *ARF* gene family. *ARF* genes, small guanine nucleotide-binding proteins, constitute a family of the RAS superfamily. This superfamily functions in a variety of cellular processes including signaling, growth, immunity, and protein transport. They have been found to be overexpressed or mutated in colon, lung, breast, colorectum, and urinary bladder cancer and acute leukemia patients (Wennerberg *et al.*, 2005).

The ARF1 protein is localized to the Golgi apparatus and has a central role in intra-Golgi transport. Multiple alternatively spliced transcript variants encoding the same protein have been found for this gene (Cukierman *et al.*, 1995). Furthermore, it has been revealed that *ARF1* expression is downregulated in human leukemia cell line in dependence of Vitamin D treatment (Savlı, 2003). However, the role of this gene in tumorigenesis is still largely undefined.

1.2.5.13. *CFL1 (Cofilin 1).* *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 cytoplasm to nucleus (Chai *et al.*, 2009).

In a recent study, it has been shown that knockdown of *CFL1* in zebrafish interfered with epibolic movement of deep cell layer, but not in the enveloping layer, and the defect could be specifically rescued by the overexpression of *CFL1* suggesting an effective role

for this gene in adhesion and cell movements (Lin *et al.*, 2010). However, this gene has not been found to be associated with cancer yet.

1.2.5.14. *PTPRF (Protein tyrosine phosphatase, receptor type, F).* The protein encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation (Chernoff, 1999).

PTPRF possesses an extracellular region, a single transmembrane region, and two tandem intracytoplasmic catalytic domains, and thus represents a receptor-type PTP. The extracellular region contains three Ig-like domains, and nine non-Ig like domains similar to that of neural-cell adhesion molecule. PTPRF was shown to function in the regulation of epithelial cell-cell contacts at adherents junctions (Harder *et al.*, 1995). 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 (Menzaghi, 2008). However, an association between this gene and cancer has not been identified yet.

1.2.5.15. *RAP1B (Member of RAS Oncogene Family).* Rap proteins are small GTPases which belong to the Ras family. Genes encoding the Rap proteins are found to be highly conserved across species. Genetic analysis of *Rap1b* function in lower eukaryotes has revealed that it is critical for development, as its loss-of-function mutations are lethal in *Drosophila* (Hariharan *et al.*, 1991). Rap1b protein shares a high degree of homology with Ras protein, especially in the effector region, and initial observations that Rap1b reverted the Ras-transformed phenotype in fibroblasts and *Xenopus* oocytes prompted investigations of the role of Rap as a Ras antagonist (Wodnicka *et al.*, 2005). While the Ras antagonist function of Rap remains controversial, there is increasing evidence that Rap signaling is involved in regulation of multiple cellular processes, including cell differentiation and adhesion (Malchinkhuu *et al.*, 2009).

1.2.5.16. *ARHGAP1 (Rho GTPase Activating Protein 1).* *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. Like other members of the Rho family, ARHGAP1 protein cycles between the GDP-bound inactive state and the GTP-bound active state in cells and is tightly controlled by a number of regulators under physiologic conditions (Lancaster *et al.*, 1994).

A recent study revealed that ARHGAP1 protein plays an important role in regulating mammalian cell genomic stability. The *ARHGAP1* knockout primary cells show reduced DNA damage repair ability; increased genomic abnormalities and induction of multiple cell cycle inhibitors, including p53, suggesting an activity for this gene in genome maintenance and cell cycle regulation (Wang *et al.*, 2007).

1.3. *Drosophila* as a Model Organism

Drosophila has been utilized as an attractive model organism for over 100 years and has contributed greatly to the understanding of genetics and development. Perhaps for this reason, the first metazoan genome to be sequenced was that of *Drosophila*. Currently, it is one of the best characterized and most widely used animal models with active research providing insights into our understanding of aging, neurodegenerative diseases, immunity, diabetes, addiction and cancer (Adams *et al.*, 2000).

Several features make *D. melanogaster* an attractive model organism for scientific research. It is a small animal, a little insect about 3 mm long, which has a short life cycle of just ten days (Figure 1.4) and grow on simple corn meal/yeast/molasses media. It is easy and not so expensive to keep large numbers of it in laboratory conditions. Furthermore, the extensive use of it as a model organism has produced an invaluable knowledge concerning its development and anatomy, as well as an extensive set of genetic tools. It has a relatively simple karyotype with only four pairs of chromosomes, and allows to establish, test and efficiently apply different transgenic and knock-out strategies using appropriate transgenic fly lines (Hedges *et al.*, 2002).

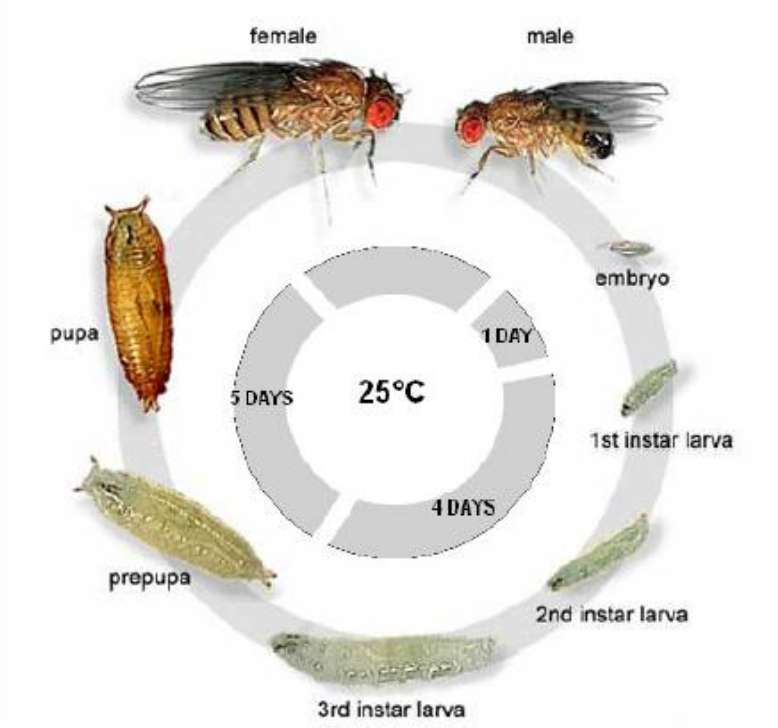

Figure 1.4. Life cycle of *D. melanogaster* (Adams *et al*., 2000).

1.3.1. Role of *Drosophila* as a Model Organism in Cancer Research

Conservation of major signaling pathways between humans and flies has made *Drosophila* an attractive model organism for cancer research, as well. With respect to cell invasion and migration, many of the morphogenetic movements required for *Drosophila* development provide great models for cancer studies *in vivo*. These developmental stages in *Drosophila* are tightly regulated by various signaling pathways such as Wnt and Notch which are also deregulated during cancer progression. Thus, *Drosophila* represents a particularly powerful genetic model for the analysis of such signaling cascades in association with cancer (Vidal *et al.*, 2006).

The developing eye of *D. melanogaster* is a good model for such studies since it is a simple and genetically well-defined organ. The growth of the eye depends on Notch activation in the dorsal–ventral organizer by its ligands Delta (human counterparts, DLL-1, -3, -4) and Serrate (human counterparts, JAGGED-1, -2). By overexpressing Delta in the eye tissue of *D. melanogaster* the large eye phenotype is produced as a tool to screen for

mutations that convert tissue overgrowths into tumours, and eventually, two attractive *in vivo Drosophila* eye cancer models are established, which are “eyeful” and “sensitized” fly models (Ferres-Marco *et al.*, 2005).

1.3.2. Eyeful *Drosophila* Cancer Model

In order to identify genes that interact with the Notch pathway and that influence growth and tumorigenesis, the Gene Search system was used to screen for genes that provoked tumours when coexpressed with Delta in the proliferating *Drosophila* eye. The eyeless-Gal4 (ey-Gal4) line was used for both eye-specific and ubiquitous induction, resulting in the transactivation of UAS-linked genes throughout the proliferating eye discs. It was through such a screen that the GS88A8 line was isolated. Generalized overexpression of Delta by ey-Gal4 (ey-Gal4>Dl) produces mild eye overgrowth. In most of the flies in which the GS88A8 line was coexpressed with Delta, tumours developed in the eyes. Moreover, in ~30% of the mutant flies, secondary eye growths were observed throughout the body, and in some flies the whole body filled up with eye tissue. These secondary eye growths had ragged borders (Figure 1.5.), indicating invasion of the mutant tissue into the surrounding normal tissue. As a consequence, the GS88A8 line was named “eyeful” (Ferres-Marco *et al.*, 2005).

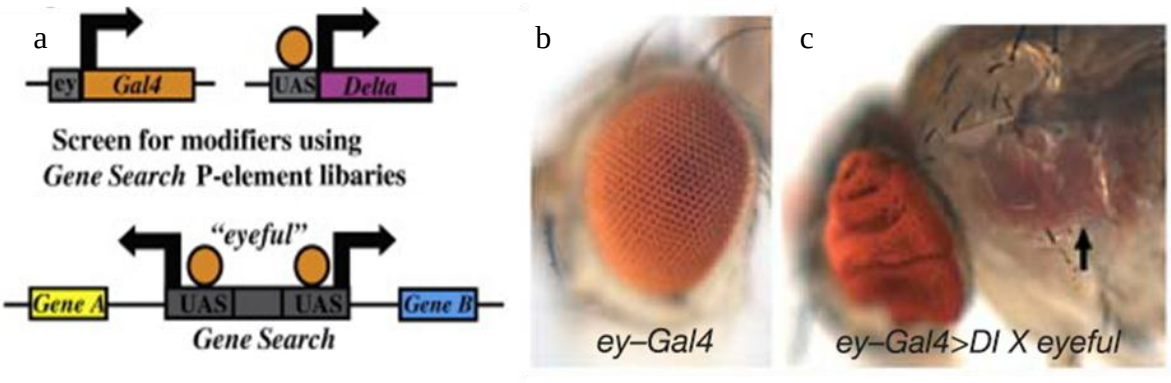

Figure 1.5. Eyeful phenotype (Ferres-Marco *et al.*, 2005).

In the eyeful flies, the overexpression of the Notch ligand Delta together with the overexpression of two Polycomb group epigenetic silencers lola and pipsqueak in the eyes induce metastatic eye tumours in flies, which is defined as the “eyeful” phenotype.

Regarding to this potential, eyeful flies are used as a novel cancer model in order to screen for genes that can further enhance or suppress the existing tumor and metastasis formations.

The eyeful phenotype has been used for the first time by Bossuyt *et al.* to screen for mutations that could further enhance the metastatic tumor formations in *Drosophila.* This screen identified *Atonal* (*ato*), encoding a transcription factor required for retinal terminal differentiation, as a crucial tumor suppressor gene. Mutations affecting *ato* dramatically enhanced tumor burden and metastasis rates, and tumors displayed elevated levels of proliferation markers such as phosphorylated histone H3, whereas overexpression of *ato* suppressed tumor and metastasis formations by inhibiting proliferation and inducing apoptosis (Bossuyt *et al.*, 2009).

1.3.3. Sensitized *Drosophila* Cancer Model

Unlike the eyeful flies which trigger metastatic eye tumours by overexpressing the Notch ligand Delta together with the Polycomb group epigenetic silencers lola and pipsqueak, sensitized flies only overexpress the Notch ligand Delta resulting in increased eye size, but no tumors or metastases (Figure 1.6.). It is a complementary cancer model of the eyeful model and enables to screen for genes which have a potential to initiate novel tumor or metastasis formations in the presence of Delta overexpression (Ferres-Marco *et al.*, 2005; Bossuyt *et al.*, 2009).

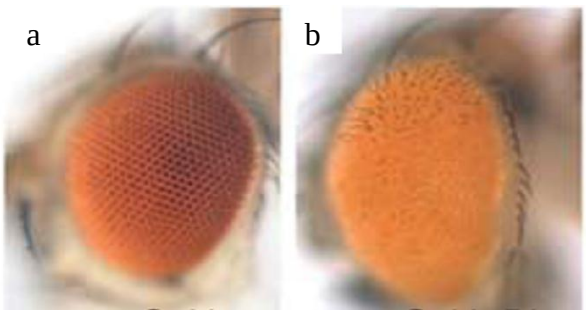

Figure 1.6. Sensitized phenotype (Bossuyt *et al.*, 2009).

Bossuyt *et al.* identified *ato* as a tumor suppressor gene in the eyeful cancer model, since its downregulation further enhanced the existing tumor and metastasis formations. They also used the sensitized model to examine whether the same gene's downregulation could lead to any tumor or metastasis formations in the sensitized model. Eventually, *ato*'s downregulation in the sensitized background resulted in many tumor and metastasis formations suggesting a tumor suppressor activity for the gene and verifying the results of the eyeful background (Bossuyt *et al.*, 2009).

1.4. RNA Interference

RNA interference (RNAi) is a form of posttranscriptional control in which the introduction of a double-stranded RNA (dsRNA) into a cell leads to specific degradation of mRNAs with complementary sequence. RNAi was first discovered in 1998 by Andrew Fire and Craig Mello in the nematode worm *Caenorhabditis elegans*, but has subsequently been found in other animal species, including *Drosophila* and humans. In 2006, Andrew Fire and Craig C. Mello shared the Nobel Prize in Physiology and Medicine for their work on RNAi which they published in 1998 (Fire *et al*, 1998).

Evolutionary, the RNAi pathway induced by dsRNA can be seen as a protective mechanism against viral attacks and to maintain genome integrity, possibly threatened by transposon elements. Indeed, the minimum components necessary for cleavage of long dsRNA into siRNA fragments and subsequent degradation of target RNAs are present in essentially all eukaryotic groups, suggesting a development that took place very early in the evolution of eukaryotic species (Shabalina *et al.*, 2008).

During the RNAi pathway, small interfering RNA molecules (siRNAs) are generated from long double-stranded RNA molecules (dsRNA) by the enzyme Dicer. These siRNA molecules are then introduced into the RNA-induced silencing complex (RISC), a high molecular weight protein complex, where the dsRNA fragments are unwound and subsequently bind to their target mRNA (Figure 1.7.) in a sequence specific manner to finally facilitate the degradation of the mRNA (Shabalina *et al.*, 2008).

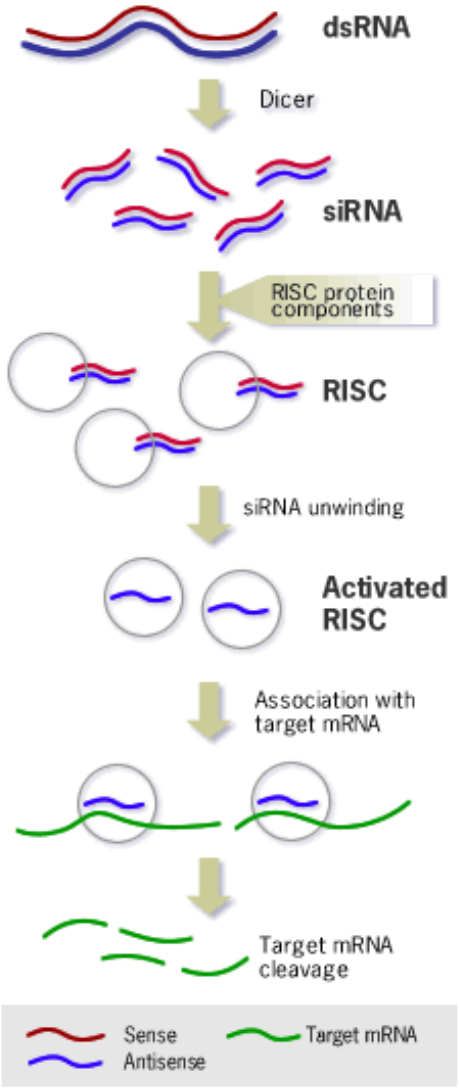

Figure 1.7. Molecular mechanism of RNAi (Shabalina *et al.*, 2008).

1.4.1. *In vivo* RNA Interference in *Drosophila*

In *Drosophila*, transgenic hairpin constructs are capable of producing gene specific RNAi, which promote inducible knockdown of genes in specific tissues via the existing GAL4/UAS system. Many lines have already been established which induce expression of the GAL4 transcription factor in a variety of tissues and patterns. When expressed, GAL4 binds its recognition site – upstream activating sequences (UAS), promoting expression of the desired target sequence. This system is often used to promote misexpression of genes in a tissue of interest, by inserting the coding region of the genes of interest downstream of

the UAS recognition site. In this bipartite system, UAS responder lines are created, and then flies are mated with GAL4 drivers of interest (Figure 1.8.) to promote expression of the gene of interest (Duffy, 2002).

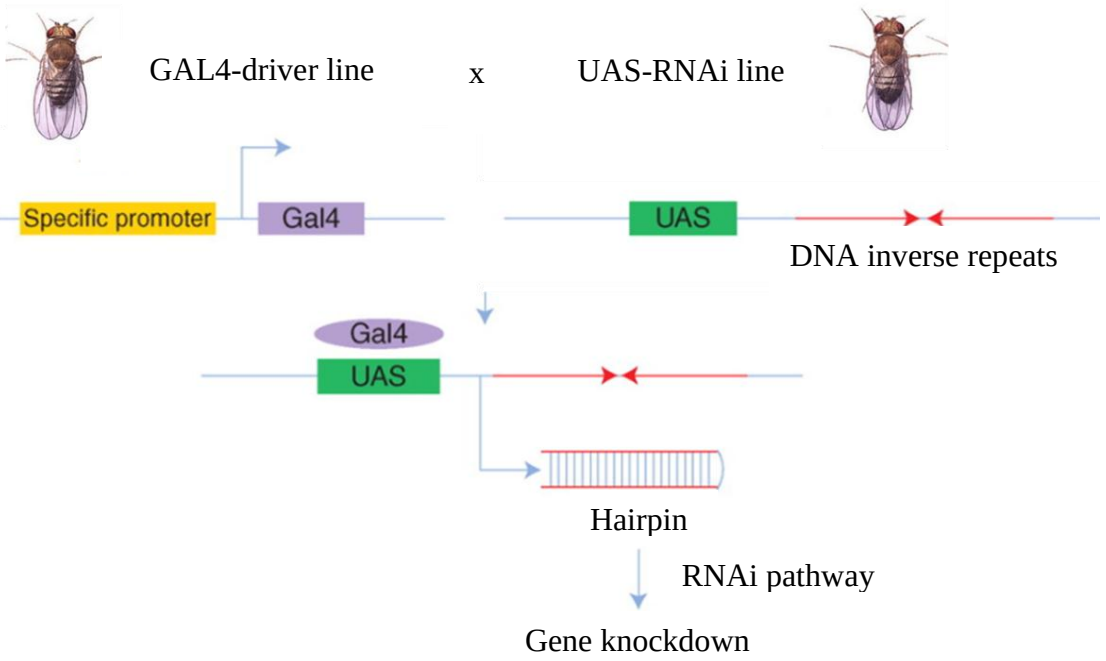

Figure 1.8. *In vivo* RNAi in *Drosophila* (Perrimon *et al.*, 2010).

Recently, the Vienna *Drosophila* RNAi Center (VDRC) has created a library of transgenic stocks with inducible RNAi constructs targeting 88.2% of the *Drosophila* genome (Dietzl *et al.*, 2007). In this library, the UAS promoter is attached to an inverted repeat sequence of approximately 300-400 bp that matches the target gene. Upon transcription, the inverted repeat folds over creating a hairpin RNA (hpRNA). The hpRNA is recognized by the RNA interference machinery of the cell and the Dicer enzyme in *Drosophila* (Dcr-2) cuts the hpRNA into short stretches of RNA of about 19-23 bps each, thereby forming a set of silencing RNAs all matching the target mRNA (Ghildiyal *et al.*, 2009). With the help of Dcr-2 and the double stranded RNA binding protein R2D2, the siRNAs are subsequently loaded onto RISC, which selects the guide strand from the siRNA with the help of Argonaute2 (Ago2). At this stage, Ago2 cleaves the passenger strand of the siRNA, forming mature RISC loaded with a single stranded RNA (guide strand). This guide strand then directs identification of mRNAs that have complementary sequence, promoting their degradation, and effectively preventing translation of target

mRNA. By this way, with the VDRC strains and existing GAL4 lines, the function of ~88% of the genes in the *Drosophila* genome can quickly be assessed in the tissue of interest (Perrimon *et al.*, 2010).

2. PURPOSE

One of the most common driving forces in HCC is the aberrant activation of the Wnt/β-catenin signaling pathway via the accumulation of mutant β-catenin in the cell. In order to identify novel targets of Wnt/β-catenin signaling, our group previously performed the overexpression of mutant β-catenin in the HCC cell line Huh7 *in vitro*. Having pursued SAGE and microarray approaches in this cell line, more than 100 genes were detected eventually with altered expression levels upon β-catenin induction. These genes are suggested to be novel targets of the Wnt/β-catenin signaling pathway which may play a role in the development and progression of HCC and other cancer types, as well. Among them, the genes mostly affected by β-catenin induction, not known to be associated with any specific cancer type and having homologs in *Drosophila* were chosen for further analysis. In this way, 16 genes were selected in total for further experimental investigations. These genes are either novel genes which are not identified yet precisely, or genes which are defined in limited levels, but their roles in cancer are largely undefined.

The aim of this study was to identify the possible effects of the selected 16 candidate genes on tumor and metastasis formations. For this purpose, an *in vivo* RNAi screen has been performed using two *Drosophila* eye cancer models, "eyeful" and "sensitized". In these cancer models, the *Drosophila* homologs of the selected genes were downregulated and the effects of these downregulations on tumor and metastasis formations were examined.

Characterization of putative Wnt/β-catenin targets and elucidation of their effects on tumor and metastasis formations may provide clues about their potential roles in cancer development. The genes detected with effective roles in tumor or metastasis formation may be identified as novel targets for the diagnostic and therapeutic processes of HCC as well as other cancer types.

3. MATERIALS

3.1. *Drosophila* Lines

Two *Drosophila* eye cancer models, eyeful and sensitized flies, containing the ey-GAL4 drivers were kindly provided by Bassem Hassan from the K. Leuven University in Belgium. The UAS-RNAi lines for each of the analyzed genes were purchased from VDRC (Table 3.1.).

Table 3.1. Fly lines used throughout the study.

Name of Line	Description
Cancer Models with GAL4 Drivers	
ey-GAL4, GS88A8, UAS-Dl/Cyo	Eyeful Eye Cancer Model
ey-GAL4, UAS-Dl/Cyo	Sensitized Eye Cancer Model
UAS-RNAi Lines	
UAS-RNAi-*Mgat1*	UAS fused to *Mgat1* dsRNA
UAS-RNAi-*Tctp*	UAS fused to *Tctp* dsRNA
UAS-RNAi-*Cam*	UAS fused to *Cam* dsRNA
UAS-RNAi-*Ip259*	UAS fused to *Ip259* dsRNA
UAS-RNAi-*Ena*	UAS fused to *Ena* dsRNA
UAS-RNAi-*Fen1*	UAS fused to *Fen1* dsRNA
UAS-RNAi-*CG2862*	UAS fused to *CG2862* dsRNA
UAS-RNAi-*Mp20*	UAS fused to *Mp20* dsRNA
UAS-RNAi-*CG11534*	UAS fused to *CG11534* dsRNA
UAS-RNAi-*Ide*	UAS fused to *Ide* dsRNA

UAS-RNAi-*Hsc70-5*	UAS fused to *Hsc70-5* dsRNA
UAS-RNAi-*Arf79f*	UAS fused to *Arf79f* dsRNA
UAS-RNAi-*YL-1*	UAS fused to *YL-1* dsRNA
UAS-RNAi-*Liprin-alpha*	UAS fused to *Liprin-alpha* dsRNA
UAS-RNAi-*Roughened*	UAS fused to *Roughened* dsRNA
UAS-RNAi-*RhoGAP68F*	UAS fused to *RhoGAP68F* dsRNA

3.2. Mammalian Cell Lines

All *in vitro* experiments are performed in the HEK293FT human embryonic kidney fibroblast cells.

3.3. General Chemicals and Kits

All chemicals used in this study were analytical grade and purchased from Sigma (St. Louis, MO, USA) and Merck (Schucdarf, Germany), unless stated otherwise in the text. Tissue culture media and solutions were purchased from Gibco (Paisley, UK), Applichem (Darmstadt, Germany), and Biochrom AG (Berlin, Germany). *In vitro* siRNA transfection system was purchased from Santa Cruz (Heidelberg, Germany). High Pure RNA Isolation Kit and Transcriptor High Fidelity cDNA Synthesis Kit were purchased from Roche (Indianapolis, USA).

3.4. Nucleic Acids

DNA molecular weight markers and deoxyribonucleotides were purchased from Fermentas (Burlington, Canada). Primers used in polymerase chain reaction were purchased from Harvard University MGH DNA Sequencing Core (Boston, USA).

3.5. Enzymes

Taq DNA Polymerase together with $MgCl_2$ (25mM), 10X reaction buffer used in this study were purchased from Fermentas (Burlington, Canada).

3.6. Electrophoresis Buffers and Solutions

50X Tris-acetic acid EDTA (TAE)	2M Tris-acetate 50Mm EDTA pH 8.5
Ethidium Bromide (EtBr)	10 mg/ml (stock solution) 30 ng/ml (working solution)

3.7. Equipments

Autoclave	Midas 55, Prior Clave, UK
Carbon dioxide tank	2091, Habaş, Turkey
Cell culture incubator	Hepa Class 100, Thermo, USA
Centrifuges	Mini Centrifuge 17307-05, Cole Parmer, USA Centrifuge 5415R, Eppendorf, USA Centrifuge, Allegra X-22, Beckman Coulter, USA
Cold room	Birikim Elektrik Soğutma, Turkey
Deep freezers	-20°C, Arçelik, Turkey -70°C, Harris, UK -86°C ULT Freezer, ThermoForma, USA
Documentation System	Gel Doc XR System, Bio-Doc, Italy
Electrophoresis Systems	Mini-sub Cell GT, BioRad, USA
Heat-blocks	DRI-Block DB-2A, Techne, UK
Heating Magnetic Stirrer	M221 Elektro-mag, Turkey

	Clifton Hotplate Magnetic Stirrer, HS31, UK
Ice Machine	Scotsman Inc., AF20, Italy
Incubators	Blue M, USA
	Weiss Gallenkamp, Plus Series, UK
Inverted Microscope	Zeiss, USA (Axio Observer, Z1)
Laboratory Bottles	Isolab, GERMANY
Laminar flow cabinet	Labcaire BH18, UK
Liquid Nitrogen Tank	Air Liquide, TR21, FRANCE
Micropipettes	Finnpipette, Thermo, USA
Microwave ovens	Philips Whirlpool, USA
	M1733N, Samsung, MALAYSIA
Pipettor	Pipetus-akku, Hirscmann Labogeraete, Germany
Power Supply	Bio-Rad, USA
Refrigerators	2082C, Arçelik, Turkey
	4030T, Arçelik, Turkey
Spectrophotometer	NanoDrop 1000, USA
Stereo Microscope	Olympus, USA (SZ61)
Thermocyclers	Applied Biosystems, GeneAmp PCR System, USA
Vortex	Vortexmixer VM20, Chiltern Scientific, UK
Water baths	TE-10A, Techne, UK
Water purification	WA-TECH Ultra Pure Water Purification System, Germany

4. METHODS

4.1. Maintenance of *Drosophila* Stocks

Stocks were reared in cylindrical vials (28.5mm × 95mm) containing fresh medium. All flies were maintained in an incubator at 25ºC with 12 hour light-dark cycle. Adult flies were placed in bottles containing fresh media, and after two to three weeks adult flies were dumped. Newly emerging adult flies were transferred into new vials. Stocks were maintained in this fashion throughout the research to assure healthy stocks and to prevent overlapping of generations.

4.2. *Drosophila* Media Recipe

73 g of cornmeal, 68 g of sugar and 15 g of nutritional yeast flakes were added into 800 ml ddH_2O and the mixture was cooked on the heat source, while stirring frequently. 15 g of agar was dissolved in 25 ml of ddH_2O and added to the yeast/cornmeal/sugar/water mix. After the mixture returned to a boil, 15 ml of propionic acid was added to the mixture. When the mixture has just begun to bubble at the edges, it was cooked for 10 more minutes stirring frequently. The final mixture was cooled to 70ºC and dispensed into vials.

4.3. Generation of Fly Lines with Desired Genes' RNAi Downregulations

Eyeful and sensitized *Drosophila* fly lines with desired gene's downregulations were generated by crossing the virgin females of eyeful and sensitized flies with the male flies of interested RNAi lines.

4.3.1. Virgin Collection

Drosophila females remain virgins for only 8-10 hours after eclosure. However, when flies are maintained at a temperature of 18ºC, development is slowed so females will not mate until 16 hours after enclosure. In order to obtain virgin *Drosophila* females, stock vials were emptied late in the afternoon making sure no adult flies remained in the vials. After, the vials were placed in an incubator of 18ºC and female flies were collected the next morning, within the first 16 hours. Since the majority of flies emerge from pupae at dawn, all female flies obtained in the morning were presumably virgins.

Moreover, it is quite easy to distinguish virgins from mature flies visually. Virgin females are much larger than older females and do not have the dark coloration of mature females. Besides, in virgin females the remains of their last meal before pupating are visible as a dark greenish spot called "meconium" (Figure 4.1.) on the underside of their abdomen. Thus, virgin females were easily distinguished by the presence of their meconium, as well.

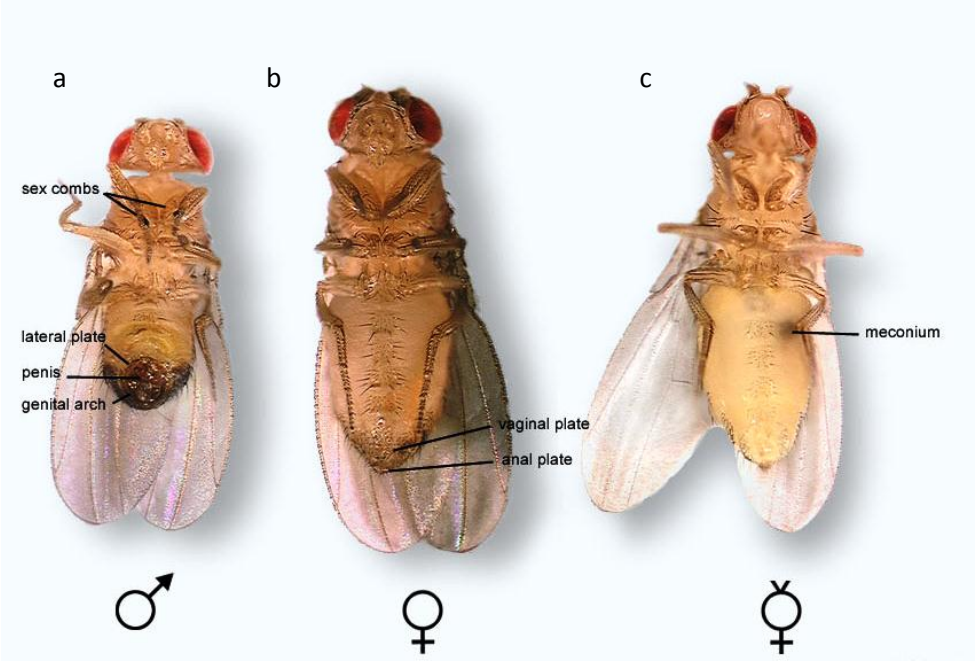

Figure 4.1. *Drosophila* adult male (a), female (b) and female virgin (c) (adapted from Greenspan, 1997).

In order to eliminate possible non-virgins from virgin females in a further step, the collected virgin females were placed in a fresh culture vial and the medium was analyzed for additional 2-3 days whether it contained any larvae. Virgin females can lay eggs, but since they are sterile there should be no larvae in the medium of virgin females.

Virgin flies were collected every morning until the desired number of virgins was reached. Virgin flies were lightly anesthetized with CO_2 and virgin males were separated from virgin females using stereo microscope. Virgin flies were maintained in polypropylene vials containing fresh media, with no more than 50 flies per vial and aged for three to four days for sexual maturation (Greenspan, 1997).

4.3.2. Crosses

To obtain eyeful and sensitized flies with desired RNAi downregulations, virgin females of eyeful and sensitized flies containing ey-GAL4 drivers were crossed with the UAS-RNAi lines of the genes of interest. All crosses were performed at 25°C. In each cross, ten male flies were crossed with 10 virgin females to generate a hybrid generation. After ten days, parental flies started dumping. In the resulting progeny, only the eyeful and sensitized flies, which do not carry the curled wing marker (Cyo) and have straight wings therefore, were selected and analyzed, since only these flies had the desired RNAi downregulations of the genes of interest.

For each gene's downregulation in the eyeful and sensitized backgrounds, three independent crosses were set up and in each cross 60 flies with desired gene's downregulation were analyzed under the stereo microscope by lightly anesthetizing them with CO_2.

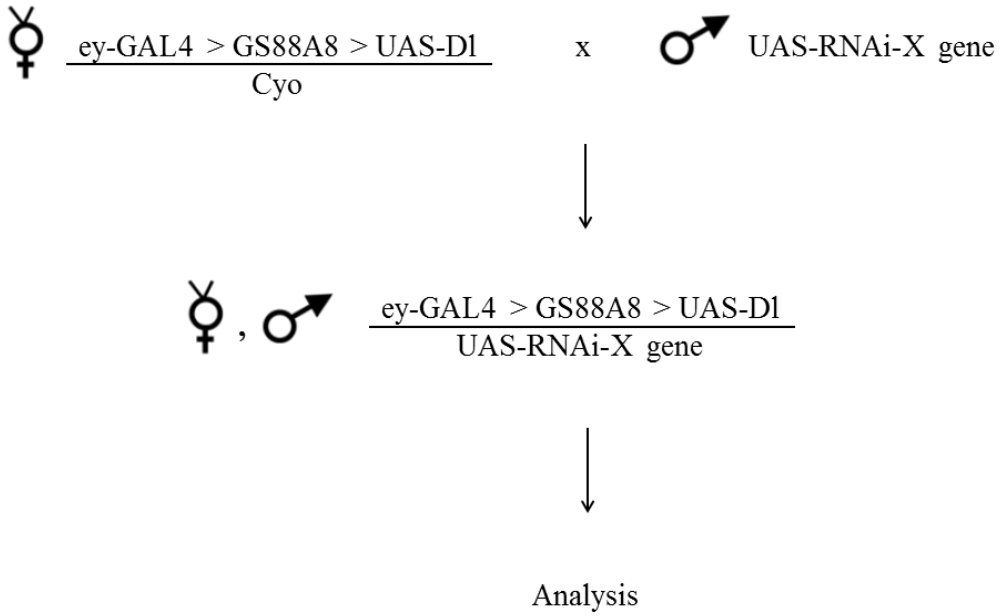

Figure 4.2. Crossing scheme for downregulation of genes with RNAi in the eyeful flies

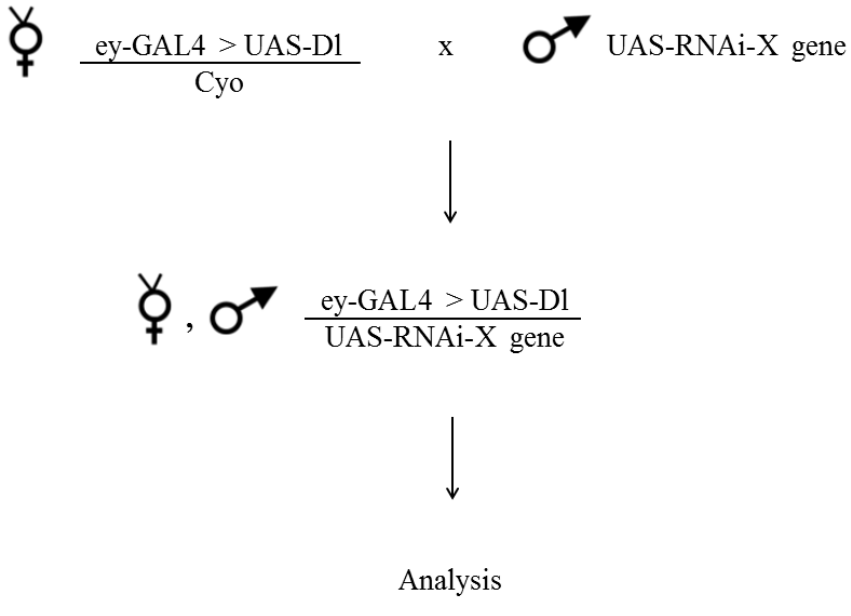

Figure 4.3. Crossing scheme for downregulation of genes with RNAi in sensitized flies

4.4. Analysis of Flies with RNAi Downregulations of Desired Genes

Eyeful and sensitized flies with desired genes' RNAi downregulations were anesthetized with CO_2 and analyzed under the stereo microscope. For each gene's downregulation, 180 flies were examined in each of the eyeful and sensitized background. In these flies, the tumor formation prevalences in the eye tissue and the metastasis formation prevalences in the entire body were analyzed. Each eye of the flies was scored separately and eyes were counted as having tumors when the eye tissue showed at least one folding. Metastasis formations could be seen as amorphous red-pigmented cells outside of the eye field.

4.4.1. Chi Square Analysis

In order to test the differences in tumor and metastasis formation prevalences upon RNAi downregulations, Chi square analysis was performed. The results of the analyzed putative target genes' downregulations were compared with the results of the negative control white gene's downregulation and the significancy of the differences between the obtained results were determined using the Chi square test.

4.4.2. RNA Extraction and cDNA Synthesis

Eyeful and sensitized flies with desired RNAi downregulations were anesthetized with CO_2 and sorted on ice. Heads of flies were removed from entire bodies and collected in RNase-free eppendorf tubes placed in liquid nitrogen. A number of ~200 heads were collected for one sample of cDNA. Heads were homogenized in lysis buffer using MagNa Lyser Green beads and a MagNa Lyser for two 15 sec pulses at 6500 rpm. RNA was purified from 350 µl homogenized tissue samples employing the Roche RNA isolation kit with standard procedure. The RNA concentration was measured with NanoDrop and 1 µg of total RNA was reverse-transcribed into cDNA using Transcriptor High Fidelity cDNA Synthesis Kit with standard protocol.

4.5. Growth Conditions of Cells and Handling

HEK293FT human embryonic kidney fibroblast cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 1% penicillin/streptomycin (complete DMEM) in an incubator at 37ºC, with 5% CO_2 and 95% air. Media were kept at 4ºC and warmed to 37ºC in a water bath before use. Containers were wiped with 70% ethanol prior to use. Cells were routinely passaged before reaching ~90% confluence. For this purpose, the growth medium was aspirated and the cells were washed once with 1X calcium and magnesium-free PBS. In order to remove the monolayer cells from the surface, the cells were treated with trypsin-EDTA solution (0.025% trypsin, 0.5mM EDTA) and incubated at 37ºC for 1 min. 5 volumes of fresh medium were added to inactivate trypsin and the suspension was pipetted gently to disperse the cells. The cells were transferred to fresh Petri dishes in a 1:5 ratio for standard passaging.

4.5.1. Thawing

One vial of frozen cell line was taken from -80ºC freezer and thawed under 40ºC (at most) running tap water or immersing the vial into 37ºC water bath. Immediately after cells are thawed, they were transferred to 100 mm culture dishes with 10 ml complete DMEM.

4.6. Transfection

In order to downregulate *Mena* in HEK293FT cells, the cells were transfected with control- and *Mena*-siRNAs using the SantaCruz transfection system according to the protocol suggested by the manufacturer.

4.7. Primers and Reverse-Transcriptase Mediated Polymerase Chain Reaction

All primers used in this study are given in the Table 4.1. For reverse-transcriptase mediated polymerase chain reaction (RT-PCR), the following reaction composition was

used: 1X Taq Buffer, 2 mM $MgCl_2$, 0.25 mM dNTP, 5% DMSO, 0.4 µM of each primer, 0.05u/µl Taq DNA polymerase (Fermentas). The PCR reaction was started with an initial denaturation step at 94ºC for 5 min. Cycling conditions of PCR were as following: denaturation step at 94ºC for 30 sec, annealing step at 55ºC for 30 sec and elongation step at 72ºC for 30 sec or 1 min. After 28-32 cycles, the PCR reaction was ended with a final elongation step at 72ºC for 7 min.

Table 4.1. Primers used in RT-PCR analyses.

Primer Name	**Primer Sequence (5'-3')**
white-F	TATTCTGCAACGAGCGACAC
white-R	GAGGTCATCCTGCTGGACAT
Axin-F	TCTGCCCAGAAGAGGTCACT
Axin-R	ATGGCTTGACAAGACCCATC
Ena-F	ATGGTGGGTCATGGTCATCT
Ena-R	TGTTGCTGTGACGGATTCAT
actin-F	ACTTCTGCTGGAAGGTGGAC
actin-R	ATCCGCAAGGATCTGTATGC
Mena-F	TCTTGGGACCACCTGCACCTC
Mena-R	CCAGCAGGGCACTCATTTCTTCC
Hes1-F	GAAGTCCTCCAAACCCATCA
Hes1-R	AGGTGCTTCACCGTCATCTC
Hes5-F	CCAGAGACACCAACCCAACT
Hes5-R	CAGAGCTTCTTTGAGGCACC
Hey1-F	ACCCAATGGACTCCACACATC
Hey1-R	TGTTCCACTGCTTGTCTGCTG
GAPDH-F	CATCCAAGGAGTGAGCCAAG
GAPDH-R	TGGAGGAAGAAATTGGAGGA

4.8. Agarose Gel Electrophoresis

DNA fragments were fractionated by horizontal electrophoresis using standard 1X TAE-based agarose gels (1% to 2%). Agarose is mixed with 1X TAE Buffer and allowed to boil in a microwave oven. After cooling for a couple of minutes, ethidium bromide was added to final concentration of 30 ng/ml and the solution was poured into the gel casting tray. Appropriate amounts of the DNA samples were mixed with 6X loading buffer to get 1X final concentration. The solidified gels were run in 1X TAE buffer at varying voltage and time depending on the size the fragments.

5. RESULTS

5.1. *In vivo* RNAi Screening in the Eyeful *Drosophila* Cancer Model

In order to identify the possible roles of the selected 16 putative Wnt/β-catenin targets in tumorigenesis, an *in vivo* RNAi screen has been performed in the eyeful *Drosophila* cancer model. The *Drosophila* homologs of the selected genes were downregulated in the eyeful model via RNAi and the effects of these downregulations on the existing tumor and metastasis prevalences in the eyeful flies were examined.

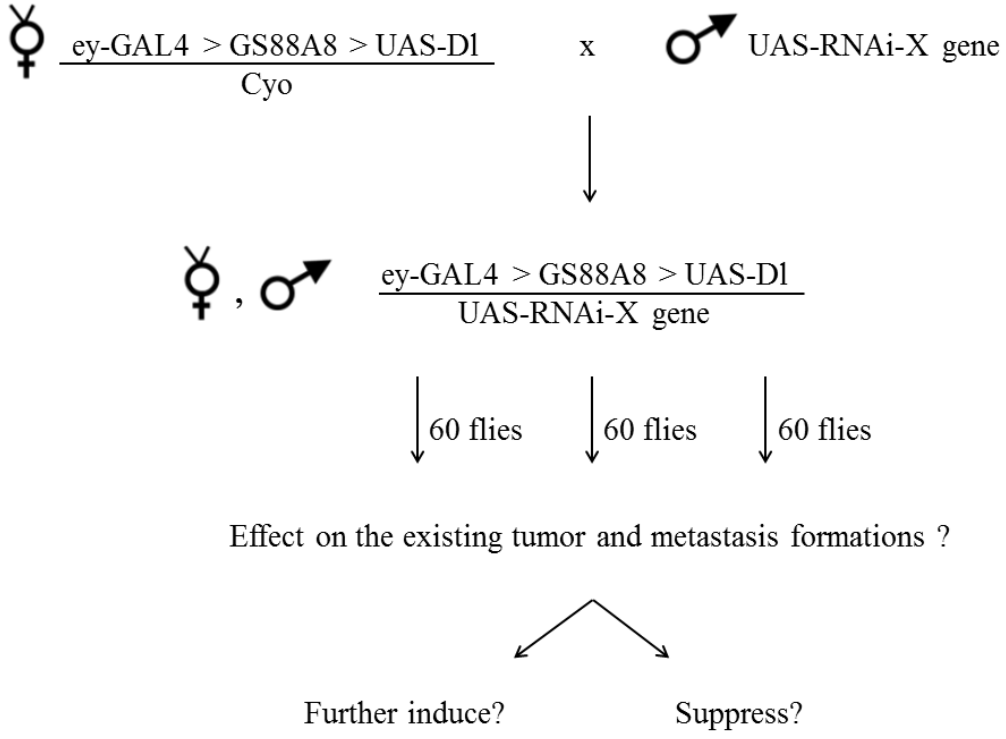

Figure 5.1. Experimental outline of the RNAi screen in the eyeful cancer model.

To downregulate the *Drosophila* homologs of the putative target genes in the eyeful cancer model, suitable transgenic UAS-RNAi fly lines of the genes of interest were crossed with the eyeful flies containing the ey-GAL4 construct (Figure 5.1), in order to enable an eye tissue-specific downregulation of the desired genes. In the resulting progeny, eyeful

flies with the downregulation of desired target genes were selected. In these selected flies, the alterations in the tumor and metastasis formation prevalences were examined.

In this RNAi screen, the *white* gene which has a function in the formation of developing eye color, but no effect on tumorigenesis was used as negative control. On the other hand, the *Axin* gene, which 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. For each gene's downregulation in the eyeful cancer model, 3 independent crosses were set up and from each cross, 180 eyeful flies with the desired downregulations were analyzed.

5.1.1. Verification of RNAi Downregulations in the Eyeful Model

In order to confirm the desired genes' downregulation in the eye tissues of the eyeful flies, RT-PCR analysis has been performed and the alterations in the expression levels of the genes of interest in the eyes of the eyeful flies has been examined.

For this analysis, the negative control *white* gene, the positive control *Axin* gene and one of the analyzed 16 putative Wnt/β-catenin target genes, *Ena*, have been selected and their expression levels upon RNAi downregulation have been investigated in comparison to the expression levels of *actin*.

In Figure 5.2., the results of the RT-PCR analysis in the eyeful flies are presented. RT-PCR analysis has revealed the decreased expression levels of the analyzed genes (*white*, *Axin* and *Ena*) in the eyes of the eyeful flies whereas the expression levels of *actin* remained unchanged confirming the gene-specific RNAi downregulation in the eyeful cancer model.

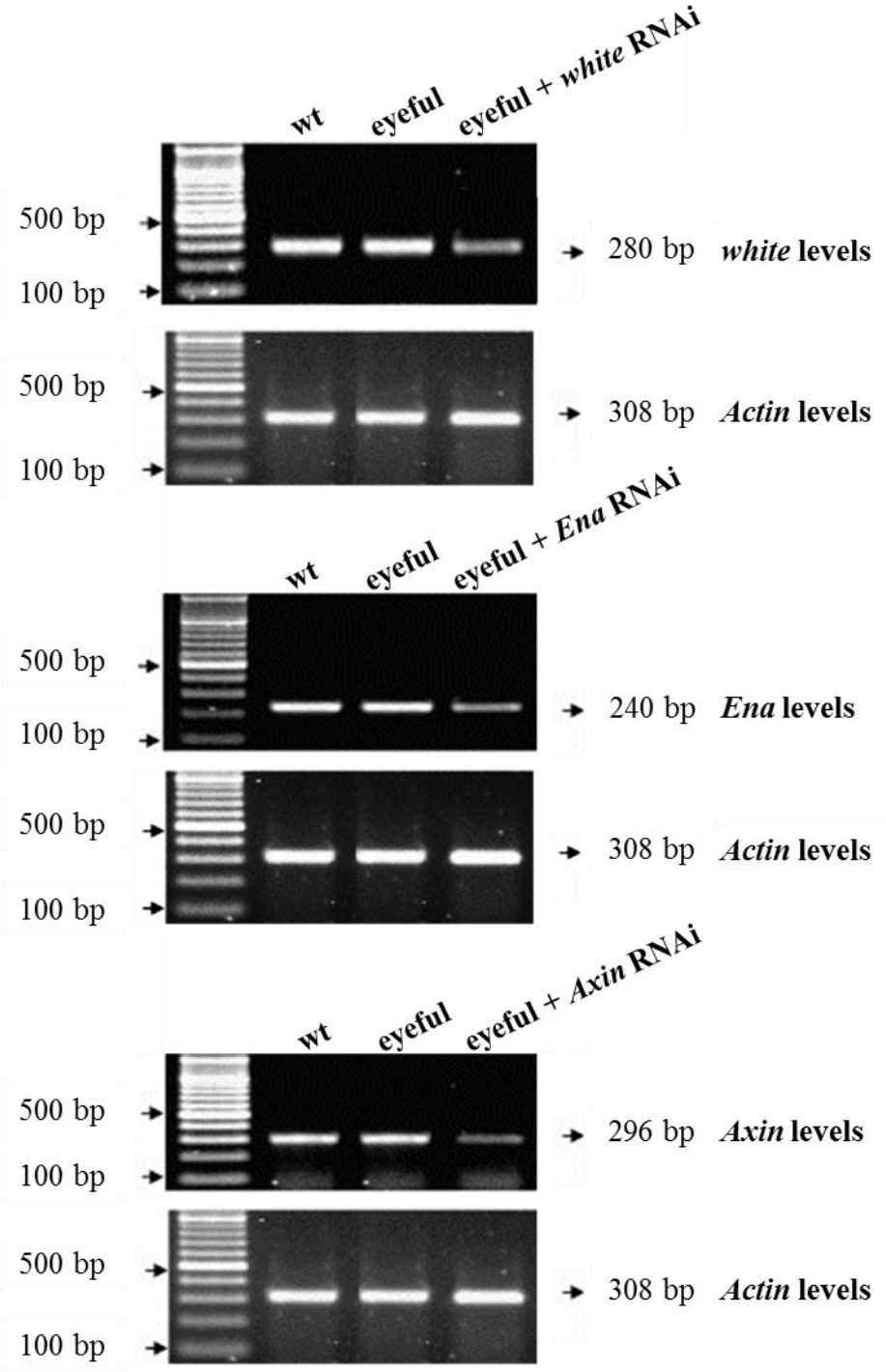

Figure 5.2. Verification of downregulation of *white*, *Ena* and *Axin* in the eyeful flies using RT-PCR analysis.

5.1.2. Analysis of Tumor Formation Prevalences in the Eyeful Model

Tumor formation prevalences were examined after downregulation of endogenous expression of genes of interest in the eyeful background. For this purpose, the eyes of 180 eyeful flies were analyzed for any tumor formation.

Several examples of the observed tumor formations are presented in Figure 5.3. Each eye of the analyzed flies was scored separately and eyes were counted as tumourous (Figure 5.3.) when the eye tissue showed at least one fold.

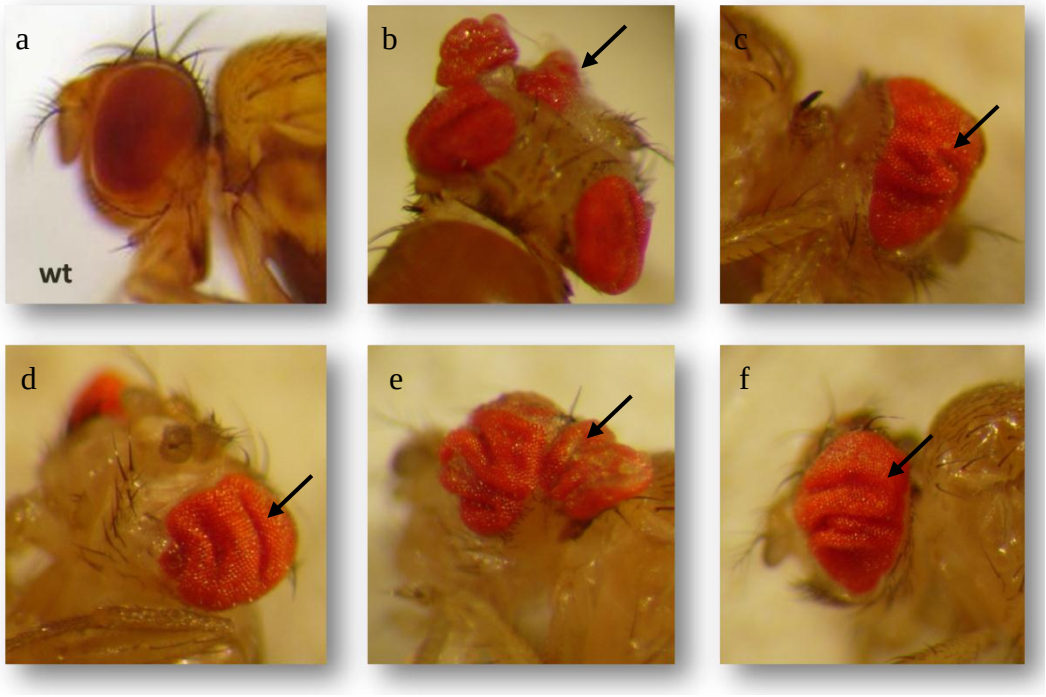

Figure 5.3. Several examples of tumor formations in the eyeful flies. Tumor formations can be seen as foldings in the eye tissue (b-f).

The tumor formation prevalences upon RNAi downregulations in the analyzed eyeful flies are presented in Figure 5.4.

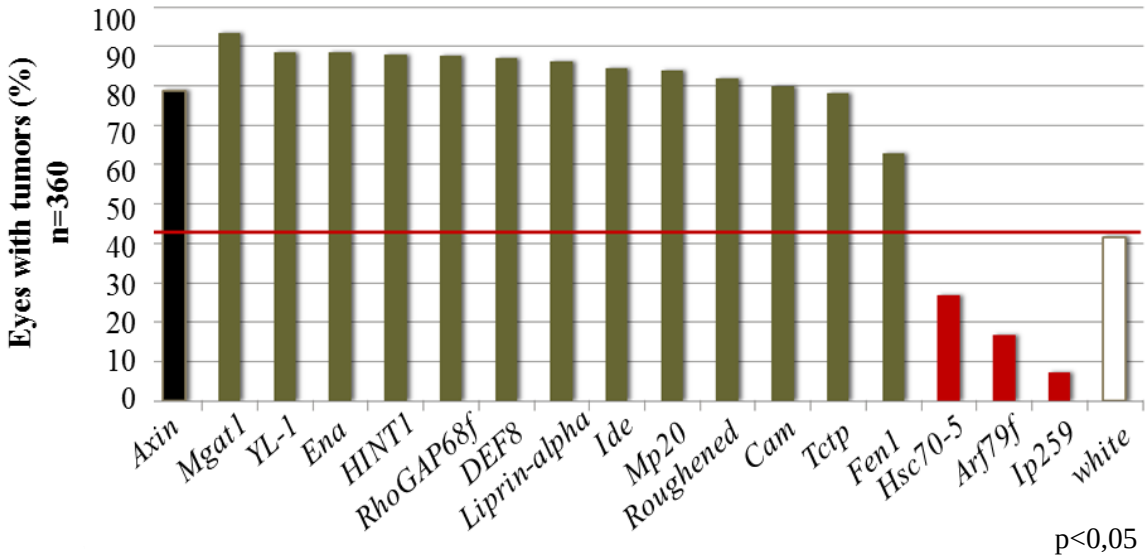

Figure 5.4. Tumor formation prevalences in the eyeful cancer model.

The downregulation of the negative control *white* gene in the eyeful flies induced tumor formation in 42% of the examined eyes (Figure 5.4., white bar). Since this gene is involved in the formation of developing eye color in *Drosophila*, its downregulation resulted in lighter eye color formation (Figure 5.5.) in comparison to wildtype flies.

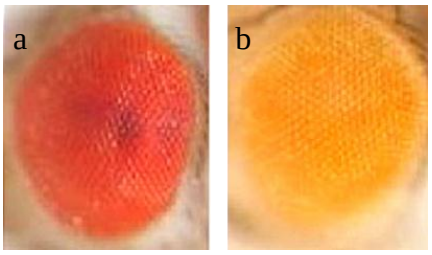

Figure 5.5. Formation of lighter eye color upon downregulation of the *white* gene in eyeful flies (b) in comparison to wildtype flies (a).

The knockdown of the positive control *Axin* gene in the eyeful flies resulted in increased frequency of eyes (79%) with tumors (Figure 5.4., black bar), compared to the negative control *white* gene. This result confirmed the known tumor suppressor activity of *Axin* in the eyeful cancer model.

The downregulation of the 16 putative Wnt/β-catenin target genes were able to affect the tumor formation prevalences in the eyeful flies, as well. Downregulation of 13 of the tested genes (*Mgat1, YL-1, Ena, HINT1, RhoGAP68f, DEF8, Liprin-alpha, Ide, Mp20, Roughened, Cam, Tctp, Fen1*) resulted in an increased tumor formation frequency ranging between 63-94% (Figure 5.4., green bars), which suggest a potential tumor suppressor activity for each of these examined genes.

On the other hand, downregulation of the remaining 3 genes (*Hsc70-5, Arf79f, Ip259*) showed decreased tumor formation prevalences ranging between 7-27% (Figure 5.4., red bars) in comparison to the negative control *white* gene, suggesting a putative oncogenic activity for these genes.

Furthermore, downregulation of the same 3 genes in the eyeful background resulted in very small- or no-eye phenotypes in 46-58% of the fly eyes suggesting an effective role for each of these genes in development, as well. Several examples of smaller- or no-eye phenotypes observed in the eyeful flies and their formation prevalences are presented in Figure 5.6 and 5.7, respectively.

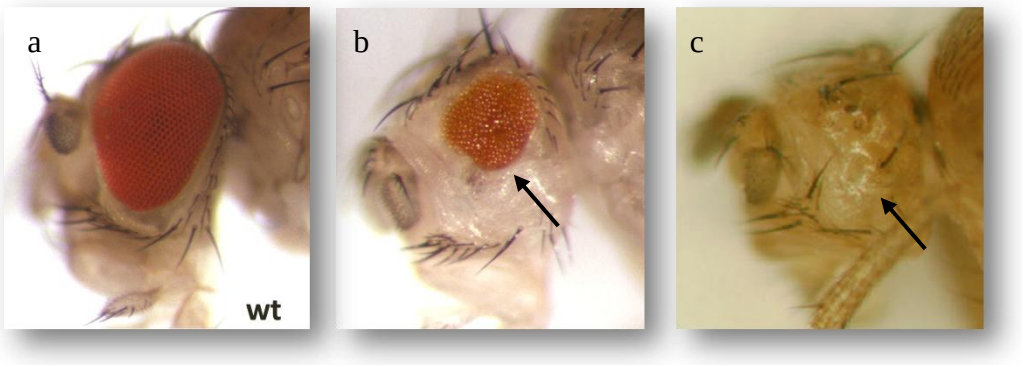

Figure 5.6. Smaller- or no-eye phenotypes in the eyeful flies.

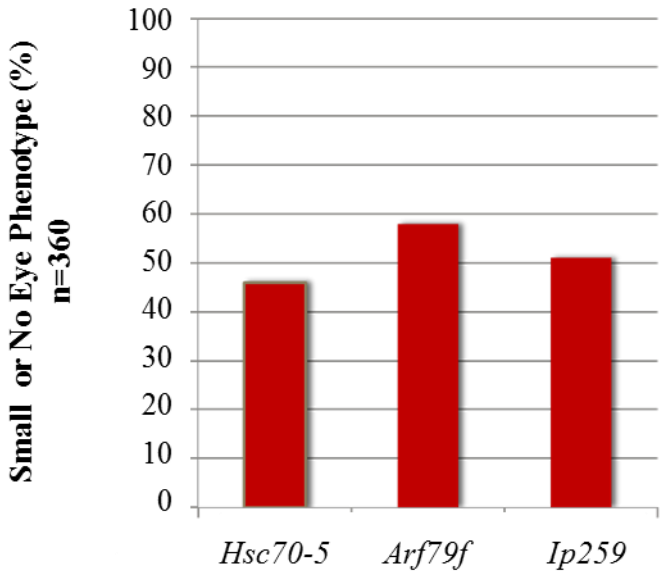

Figure 5.7. Eyeful flies showing small- or no-eye phenotype upon RNAi knockdowns.

5.1.3. Analysis of Metastasis Formation Prevalences in the Eyeful Model

After the examination of the tumor formation prevalences upon downregulation of candidate genes in the eyeful background, metastasis formation frequencies have been analyzed in the same 180 eyeful flies in a further step. The metastasis formation prevalences in these flies and several examples of the observed metastasis formations are presented in the Figure 5.8. and 5.9, respectively. Metastasis could be seen as amorphous red pigmented cells outside of the eye field in the flies. In the dorsal part, ventral part or even in the neck of the flies (Figure 5.8.) metastasis could be triggered.

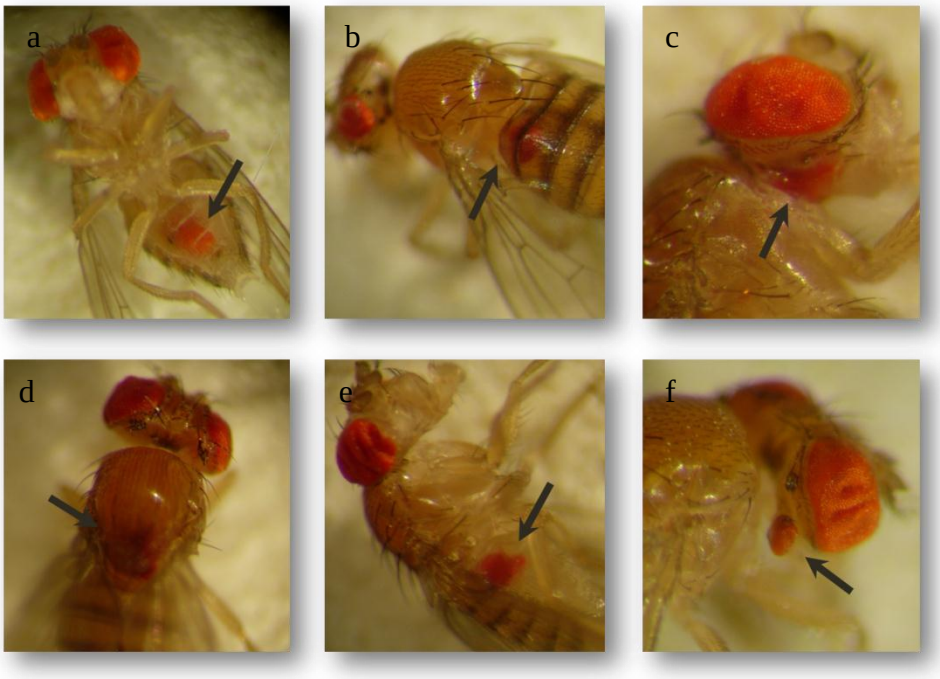

Figure 5.8. Several examples of metastasis formations in the eyeful flies.

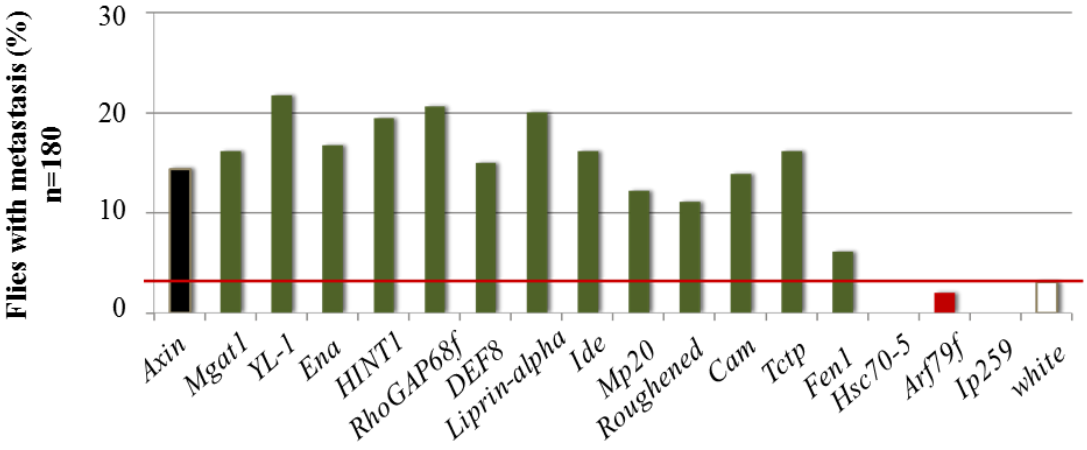

Figure 5.9. Metastasis formation prevalences in the eyeful cancer model.

The downregulation of the negative control *white* gene in the eyeful flies showed metastasis formations in 3% of the examined flies (Figure 5.9., white bar), whereas the positive control *Axin* gene induced metastasis formations in 14% of the flies (Figure 5.9., black bar), when downregulated.

The downregulations of the 16 putative Wnt/β-catenin target genes were able to further induce or suppress the existing metastasis formation frequency in the eyeful flies, as well. The downregulation of 13 genes (*Mgat1, YL-1, Ena, HINT1, RhoGAP68f, DEF8, Liprin-alpha, Ide, Mp20, Roughened, Cam, Tctp, Fen1*), which showed increased tumor formation prevalences, resulted also in increased metastasis formation frequencies ranging between 6-21% (Figure 5.9., green bars) in the eyeful flies, suggesting a potential tumor suppressor activity for these genes.

On the other hand, the downregulations of the 3 candidate genes (*Hsc70-5, Arf79f, Ip259*), which showed decreased tumor formation prevalences, were able to totally (*Hsc70-5, Ip259*) or partially (*Arf79f*) suppress the metastasis formation in the eyeful background (Figure 5.9.) suggesting an oncogenic function for these genes.

5.2. *In vivo* RNAi Screening in the Sensitized *Drosophila* Cancer Model

The downregulation of the 16 candidate genes were able to further induce or suppress the existing tumor and metastasis formations in the eyeful cancer model. In order to further investigate whether the same genes have a potential to induce novel tumor and/or metastasis formation in the sensitized background, these genes were downregulated in the sensitized *Drosophila* cancer model via RNAi and the effects were examined.

To downregulate the *Drosophila* homologs of the candidate genes, suitable transgenic RNAi fly lines of the genes of interest were crossed with the sensitized flies containing the ey-GAL4 drivers (Figure 5.10) in order to enable an eye tissue-specific downregulation of the candidate genes in the sensitized flies. In the resulting progeny, sensitized flies carrying the RNAi construct of the target genes were selected. In these selected flies, tumor and metastasis formation prevalences were examined.

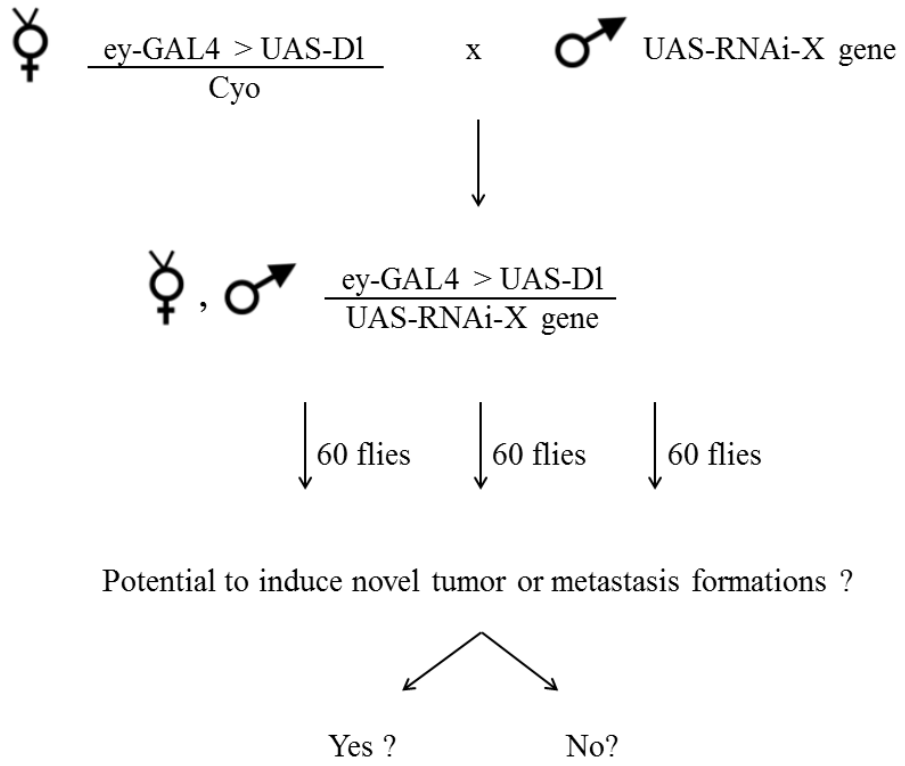

Figure 5.10. Experimental outline of the RNAi screen in the sensitized cancer model.

In this screen, again the *white* and the *Axin* genes were used as negative and positive control, respectively. For the analysis of each gene in the sensitized cancer model, 3 independent crosses were set up and from each cross, 180 flies were analyzed.

5.2.1. Verification of RNAi Downregulations in the Sensitized Model

In order to confirm the downregulation of the candidate genes in the eyes of the sensitized flies, RT-PCR analysis has been performed and changes in expression levels of the candidate genes have been examined. For this examination, the negative control *white* gene, the positive control *Axin* gene and one of the candidate genes, *Ena*, have been selected and their expression levels upon RNAi downregulation in the eyes of sensitized flies have been analyzed in comparison to the expression levels of *actin*.

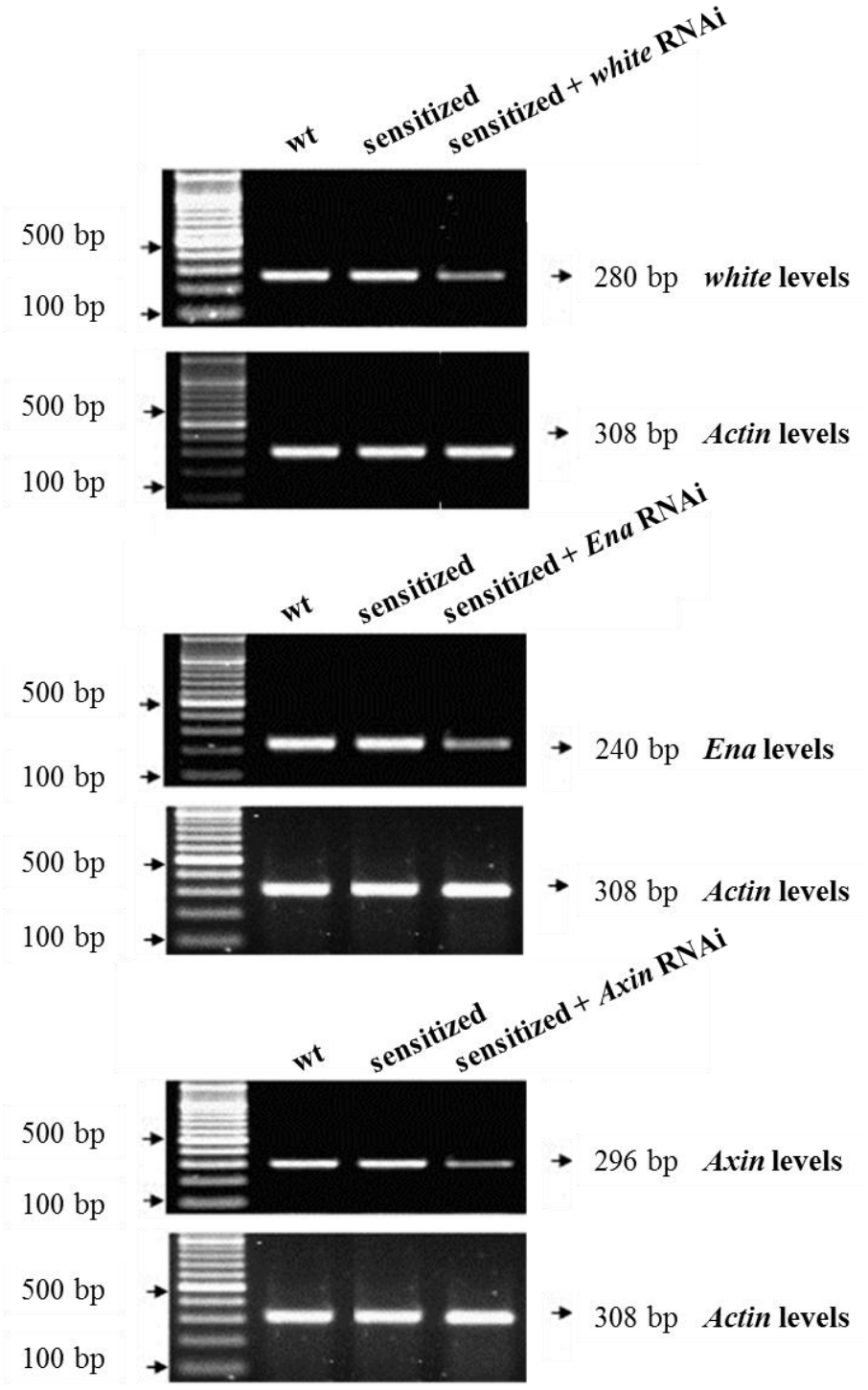

Figure 5.11. Verification of downregulation of candidate genes in the sensitized background with RT-PCR analysis.

In Figure 5.11., the results of the RT-PCR analysis in sensitized flies are presented. RT-PCR analysis has revealed the decreased expression levels of the examined genes (*white*, *Axin* and *Ena*) in the eyes of the sensitized flies, whereas the expression levels of

actin remained unchanged, confirming the gene-specific RNAi downregulation in the sensitized cancer model.

5.2.2. Analysis of Tumor Formation Prevalences in the Sensitized Model

In the sensitized flies the effects of the downregulation of the candidate genes on tumor formation were examined. For this purpose, eyes of the 180 sensitized flies with the specific knockdown of the gene of interest were analyzed for potential tumor formation. Each eye of the analyzed flies was scored separately and eyes were counted as having a tumour when the eye showed at least one fold.

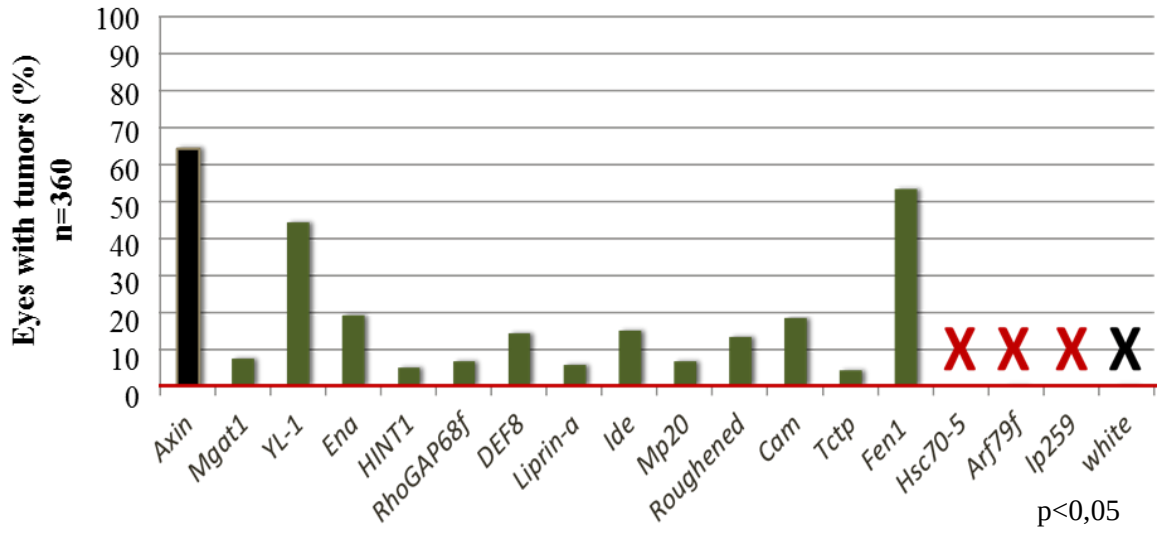

Figure 5.12. Tumor formation prevalences upon RNAi downregulations in the sensitized cancer model.

In Figure 5.12., the tumor formation prevalences in the analyzed sensitized flies are presented. The downregulation of the negative control *white* gene in the sensitized background could not induce any tumor formation, whereas the downregulation of the positive control *Axin* gene, triggered tumor formation in 64% of the analyzed sensitized flies (Figure 5.12), confirming its tumor suppressor function.

The analyzed downregulation of 13 candidate genes (*Mgat1, YL-1, Ena, HINT1, RhoGAP68f, DEF8, Liprin-alpha, Ide, Mp20, Roughened, Cam, Tctp, Fen1*), which showed increased tumor and metastasis frequencies in the eyeful background, were able to induce tumor formation in the sensitized cancer model as well with prevalences ranging between 4-53% (Figure 5.12., green bars). These data suggest a tumor suppressor function for each of these genes and confirms the results obtained in the eyeful background.

On the other hand, downregulation of the remaining 3 candidate genes (*Hsc70-5, Arf79f, Ip259*), which resulted in decreased tumor and metastasis formation prevalences in the eyeful background, did not induce any tumor formation in the sensitized flies (Figure 5.12.), suggesting an oncogenic function for these genes and supporting the results of the eyeful background.

Furthermore, downregulation of the same 3 genes resulted in very small or no eye phenotype in 46-58% of the fly eyes in the sensitized background (Figure 5.13.), as well, suggesting an effective role for each of these genes in development.

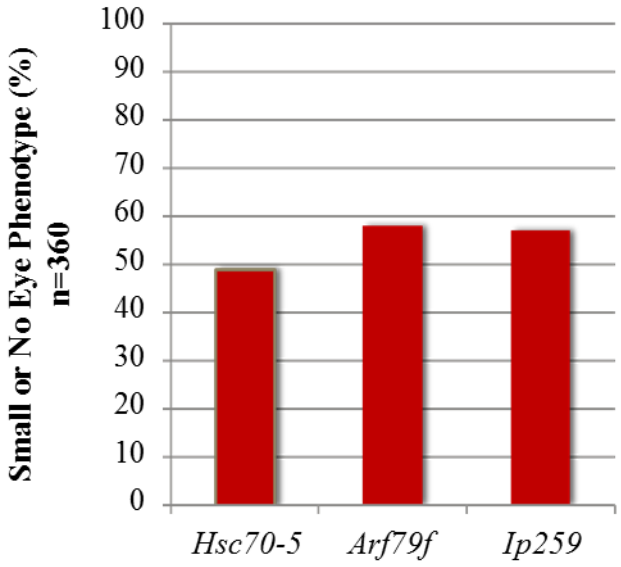

Figure 5.13. Sensitized flies showing small- or no-eye phenotype upon RNAi knockdown.

5.2.3. Analysis of Metastasis Formation Prevalences in the Sensitized Model

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

In Figure 5.14., the metastasis formation prevalences in the analyzed sensitized flies are presented. The downregulation of the negative control *white* gene in the sensitized flies showed no metastasis formation. However, the positive control *Axin* gene was also not able to induce metastasis formation in the sensitized flies, when downregulated.

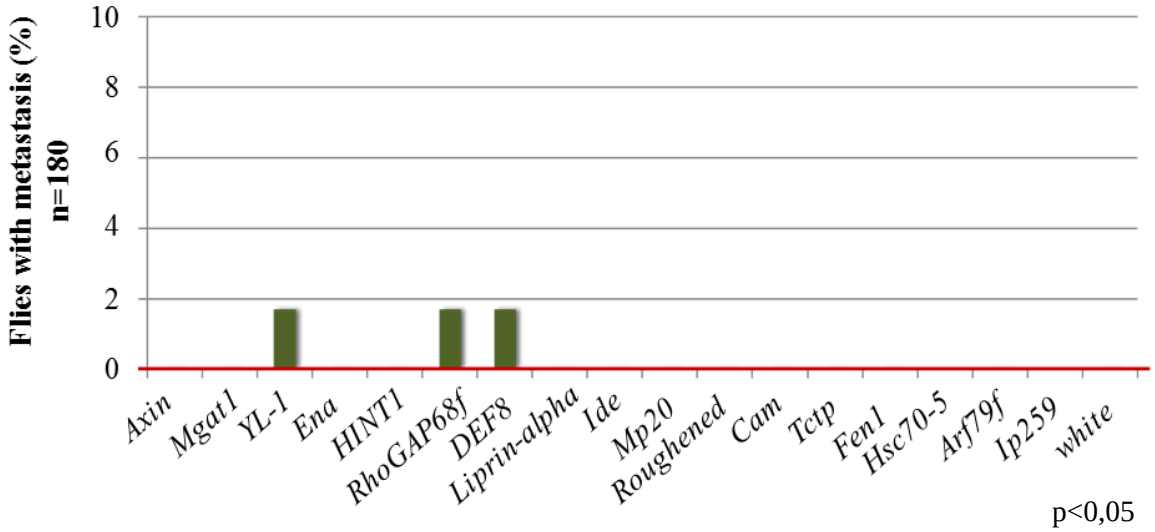

Figure 5.14. Metastasis formation prevalences upon RNAi downregulation in the sensitized cancer model.

On the other hand, the downregulation of the 3 candidate genes (*YL-1*, *RhoGAP68f*, *DEF8*), which resulted in enhanced tumor and metastasis formation percentages in the eyeful background and induced tumor formation in the sensitized background, were able to induce metastases (Figure 5.14), as well. However, the other 13 candidate genes did not trigger any metastases formations (Figure 5.14) in the sensitized flies, when downregulated.

5.3. Total Results of the *in vivo* RNAi Screening

According to the results obtained from the eyeful and sensitized backgrounds, the analyzed genes can be examined in two groups: In the first group, downregulation of the 13 putative Wnt/β-catenin target genes (*Mgat1, YL-1, Ena, HINT1, RhoGAP68f, DEF8, Liprin-alpha, Ide, Mp20, Roughened, Cam, Tctp, Fen1*) were able to enhance the existing tumor formation prevalences in the eyeful flies and to induce novel tumor formations in the sensitized flies. Furthermore, downregulation of the same genes increased the existing metastasis formation prevalences in the eyeful flies and three of them were able to trigger novel tumor formation in the sensitized background, as well, when downregulated. The data of the sensitized background are compatible with the data obtained from the eyeful background (Table 5.1) and both of them suggest a potential tumor suppressor function for each of these genes.

Table 5.1. Genes with suggested tumor suppressor functions. "+" indicates the enhancement and "-" denotes the suppression of tumor or metastasis formations, whereas "x" implies non-affected metastasis formation prevalences.

	Human Gene	***Drosophila* Homolog**	***Eyeful Cancer Model***		***Sensitized Cancer Model***	
			Tumor	***Metastasis***	***Tumor***	***Metastasis***
1.	*MGAT1*	*Mgat1*	+	+	+	*x*
2.	*TPT1*	*Tctp*	+	+	+	*x*
3.	*CALM3*	*Cam*	+	+	+	*x*
4.	*DEF8*	*CG11534*	+	+	+	+
5.	*MENA*	*Ena*	+	+	+	*x*
6.	*FEN1*	*Fen1*	+	+	+	*x*
7.	*HINT1*	*CG2862*	+	+	+	*x*
8.	*CNN3*	*Mp20*	+	+	+	*x*
9.	*RAP1B*	*Roughened*	+	+	+	*x*

10.	*IDE*	*Ide*	+	+	+	*x*
11.	*CFL-1*	*YL-1*	+	+	+	+
12.	*ARHGAP1*	*RhoGAP68f*	+	+	+	+
13.	*PTPRF*	*Liprin-alpha*	+	+	+	*x*

In the second group of genes, downregulation of the 3 putative Wnt/β-catenin target genes (*Hsc70-5, Arf79f, Ip259*) decreased the existing tumor formation prevalences in the eyeful flies and did not trigger novel tumor formations in the sensitized flies. The same downregulation decreased also the existing metastases prevalences in the eyeful flies (Table 5.2.) and did not induce novel metastases in the sensitized flies.

In addition to that, these downregulations resulted in smaller or no eye phenotype in approximately half of the analyzed eyeful and sensitized flies. Also for these genes, the data of the sensitized background are compatible with the data obtained from the eyeful background (Table 5.2.) suggesting a potential oncogenic function or a putative developmental role for each of these genes

Table 5.2. Genes with suggested oncogenic or developmental functions. "-" denotes the suppression of tumor or metastasis formations, whereas "x" implies non-affected tumor or metastasis formation prevalences.

	Human Gene	***Drosophila* Homolog**	***Eyeful Cancer Model***		***Sensitized Cancer Model***	
			Tumor	***Metastasis***	***Tumor***	***Metastasis***
1.	*Mortalin*	*Hsc70-5*	-	-	*x*	*x*
2.	*ARF1*	*Arf79f*	-	-	*x*	*x*
3.	*TINP1*	*Ip259*	-	-	*x*	*x*

5.4. *MENA* as a Novel Candidate Gene in the Possible Wnt-Notch Crosstalk?

Having pursued SAGE and microarray approaches, we previously found *MENA* to be upregulated upon β-catenin induction in the HCC cell line Huh7, suggesting a role for this gene as a transcriptional target of the Wnt/β-catenin pathway. Several further studies performed in our laboratory support this putative role of *MENA*, as well.

Using luciferase promoter assay, it has been confirmed that the putative TCF4-binding elements conserved among mammalian homologues of *MENA* are indeed functional and regulated by the Wnt/β-catenin pathway. The inhibition of GSK3β, the negative regulator of the Wnt/β-catenin pathway, resulted in increased *MENA* mRNA levels concurrently with the cytosolic accumulation and nuclear translocalization of β-catenin. Furthermore, β-catenin has been found to be directly interacting with the *MENA* promoter in Huh7 and HEK293 cells, as well as adult mouse brain and liver tissues (Najafov *et al.*, 2012). These data support a role for *MENA* as a transcriptional target of the Wnt/β-catenin signaling pathway.

In several cancer types, Wnt/β-catenin pathway is known to be overactivated and as a possible transcriptional target of this signaling, *MENA* has been found to be upregulated in several tumors, as well, suggesting a tumorigenesis-promoting role for this gene in carcinogenesis. In consistence with these findings, previous studies performed in our laboratory showed that *MENA* mRNA levels are strongly upregulated in several human solid brain tumors (2- to 6-fold increase) and that *MENA* mRNA levels and β-catenin expression correlates in tested cancer cell lines (Najafov *et al.*, 2012).

On the other hand, the knockdown of the *Drosophila* homolog *MENA* was able to enhance the Notch-driven tumor and metastasis formation prevalences in the eyeful flies and to trigger novel tumor formations in the sensitized flies, in the presence of overactivated Notch signaling. These data together identify *MENA* as a novel Wnt/β-catenin pathway target which plays a role in repressing Notch-mediated tumorigenesis in contrast to the previously reported role for this gene as a tumorigenesis-promoting protein.

In order to investigate the effect of *MENA* downregulation on the Notch signaling pathway, we downregulated *MENA* in HEK293FT human embryonic kidney fibroblast cells and analyzed the expression levels of 3 different Notch pathway targets, *Hes1*, *Hes5* and *Hey1*, by performing RT-PCR analysis.

In Figure 5.15., the results of the RT-PCR analysis are presented. The expression levels of *MENA* were found to be downregulated in the HEK293FT cells transfected with *MENA* siRNA in comparison to the cells transfected with control siRNA. In the same cells, the expression levels of three direct Notch signaling targets, *Hes1*, *Hes5* and *Hey1*, were examined and found to be increased upon *MENA* downregulation (Figure 5.15), whereas the expression levels of *GAPDH* remained unchanged.

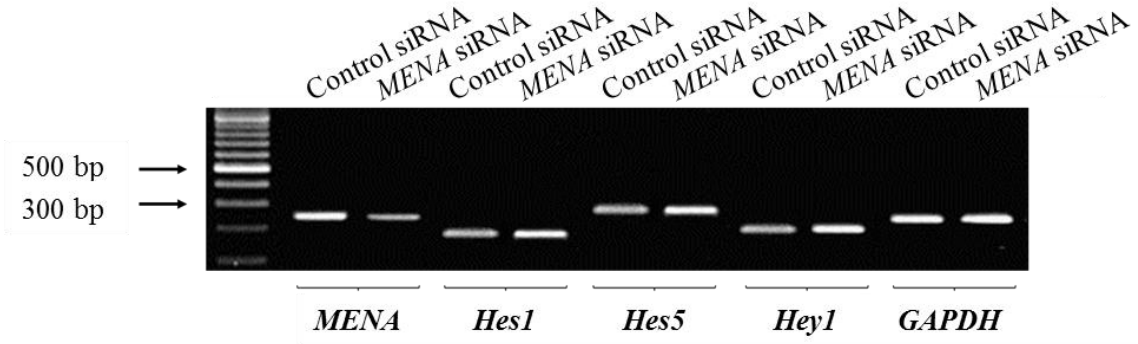

Figure 5.15. RT-PCR analysis of the Notch target gene expression levels upon *MENA* downregulation in the HEK293FT cell line.

RT-PCR analysis revealed a putative role for *MENA* as a repressor of the Notch signaling pathway. These findings put *MENA* at a critical point of a possible crosstalk between Wnt/β-catenin and Notch signaling pathways and provide a novel example of a Wnt target that may play an effective role in the suppression of Notch signaling.

6. DISCUSSION

6.1. Identification of Putative Wnt/β-catenin Signaling Targets as Potential Tumor Suppressor Genes or Oncogenes

The canonical Wnt/β-catenin signaling is an evolutionary conserved pathway which is involved in various events during embryonic development, such as axis formation, cellular proliferation, differentiation and morphogenesis. Apart from its role in development, 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 hepatocellular carcinoma. Those characteristics of the Wnt/β-catenin signaling pathway makes the pathway itself and its targets important key subjects for cancer studies.

Beta-catenin is the key mediator of the Wnt/β-catenin signaling pathway, that forms a complex with the TCF/LEF family of transcription factors in the presence of Wnt ligand and activates the transcription of several target genes such as *Axin*, *c-myc* and *Cyclin D1*, which are involved in cell proliferation, differentiation or morphogenesis. In 19-41% of human HCC samples, this key molecule is reported to be mutated resulting in nuclear accumulation of β-catenin, and thereby mimicking the Wnt stimulation.

In order to identify novel transcriptional targets of the Wnt/β-catenin pathway, a microarray and SAGE screen was carried out in our laboratory. Consequently, a number of genes were determined to be either upregulated or downregulated significantly upon mutated β-catenin accumulation 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 are found to be closely related with tumorigenesis, the putative target genes of the canonical Wnt/β-catenin

signaling pathway, which are diffentially expressed upon β-catenin induction, may play effective roles in cancer. Our aim in this study was to identify the possible effects of selected 16 candidate genes on tumor and metastasis formations and characterize their potential roles in cancer. For this purpose, an *in vivo* RNAi screen was performed in two *Drosophila* eye cancer models, "eyeful" and "sensitized". In these cancer models, the *Drosophila* homologs of the selected 16 genes were downregulated and the effects of these downregulation on tumor and metastasis formations were examined.

In the eyeful cancer model, the overexpression of the Notch ligand Delta together with the overexpression of two epigenetic silencers lola and pipsqueak promotes tumor formations in approximately 50% of flies and metastasis formation in 1-2% of flies. In this background, the 13 analyzed putative Wnt/β-catenin target genes (*MGAT1, CFL1, HINT1, DEF8, PTPRF, MENA, ARHGAP1, IDE, CNN3, RAP1B, CALM3, TPT1* and *FEN1*) further enhanced these tumor and metastasis rates, when downregulated; whereas the other 3 genes' (*Mortalin, ARF1* and *TINP1*) downregulations, suppressed the existing tumor and metastases totally or partially and resulted in smaller- or no-eye phenotypes in approximately half of the flies.

By ignoring the overexpression of lola and pipsqueak from the tumor-inducing background of eyeful flies in a further step, the effects of downregulation of the same genes were examined in the sensitized cancer model, where the overexpression of the Notch ligand Delta alone is not able to trigger tumor or metastasis formation. In this background, similar to the results in the eyeful background, downregulation of 13 candidate genes induced novel tumor formations, while 3 of them were able to promote metastasis formation, when downregulated. On the other hand, downregulation of 3 genes did not trigger any tumor or metastasis formation and resulted again in smaller- or no-eye phenotypes in approximately half of the flies in the same background.

In consistence with each other, the results of the eyeful and sensitized backgrounds together suggest a tumor suppressor function for the analyzed 13 candidate genes (Table 6.1.) and an oncogenic or a developmental function for the remaining 3 genes.

Table 6.1. The analyzed putative Wnt/β-catenin target genes with total results.

	Human gene	**Expression upon β-catenin induction**	***In vivo* RNAi screen**	**Literature**
1.	*MGAT1*	Downregulated	Tumor suppressor	N-glycan synthesis
2.	*TPT1*	Downregulated	Tumor suppressor	Cell proliferation, antiapoptosis
3.	*CALM3*	Downregulated	Tumor suppressor	Ca^{+2} regulator, mitosis
4.	*TINP1*	Upregulated	Oncogene/ Development	Cell cycle
5.	*MENA*	Upregulated	Tumor suppressor	Cell motility, adhesion
6.	*FEN1*	Downregulated	Tumor suppressor	Genomic stability, DNA repair
7.	*HINT1*	Downregulated	Tumor suppressor	Tumor suppressor?
8.	*CNN3*	Downregulated	Tumor suppressor	Cytoskeleton
9.	*DEF8*	Downregulated	Tumor suppressor	Unknown
10.	*IDE*	Downregulated	Tumor suppressor	Insulin metabolism
11.	*Mortalin*	Upregulated	Oncogene/ Development	Binding partner of p53
12.	*ARF1*	Upregulated	Oncogene/ Development	Golgi transport
13.	*CFL1*	Downregulated	Tumor suppressor	Cell adhesion, cell migration
14.	*PTPRF*	Downregulated	Tumor suppressor	Cell contact
15.	*RAP1B*	Downregulated	Tumor suppressor	Ras antagonist?
16.	*ARHGAP1*	Upregulated	Tumor suppressor	Genomic stability, DNA repair

In comparison to the previous SAGE and microarray results, these findings are highly compatible with the detected expression levels of the analyzed genes in the HCC cell line Huh7 upon β-catenin induction. For instance, the 3 candidate genes (*Mortalin, ARF1* and *TINP1*), which are characterized as potential oncogenes in the RNAi screen in *Drosophila*, were found to be upregulated in the HCC cell line upon β-catenin induction, supporting the oncogenic roles of these genes. Furthermore, 11 potential target genes (*MGAT1, CFL1, HINT1, DEF8, PTPRF, IDE, CNN3, RAP1B, CALM3, TPT1* and *FEN1*), which are characterized as potential tumor suppressors in the RNAi screen, were found to be downregulated upon β-catenin induction (Table 6.1.), confirming the tumor suppressor functions for these genes.

However, the other 2 analyzed genes (*MENA* and *ARHGAP1*) showed inconsistent results in the RNAi screen in comparison to the SAGE and microarray results. Although these two candidate genes were found to be upregulated in the HCC cell line upon β-catenin induction, their downregulations were able to enhance the Notch induced tumorigenesis in *Drosophila* (Table 6.1.). These contradictory findings may give clues about an inhibitory effect of the Wnt/β-catenin signaling on the Notch pathway via these two genes. The dysregulation of this inhibitory crosstalk between the Wnt and Notch signaling pathways via these two genes may trigger tumorigenesis, mimicking the increased tumor and metastasis rates upon their downregulation in the *Drosophila* cancer models.

In several studies, some of these examined 16 genes have been characterized as being involved in various processes such as cell cycle control, proliferation, cell-motility or genomic instability giving clues about their potential roles in the signaling network of tumorigenesis, whereas some of them have not been identified precisely yet. For instance, one of these genes, *TINP1* has been suggested to play a role as a cell growth regulator in cell cycle progression, since its overexpression promoted and its knockdown attenuated the cell growth by regulating the cell cycle in G1/S transition (Zhang *et al.*, 2010). *ARHGAP1* knockout primary cells showed reduced DNA damage repair ability and increased genomic abnormalities suggesting an essential role for this gene in the maintenance of genomic stability (Wang *et al*., 2007). On the other hand, *MENA* has been identified as an important

component of the actin-regulatory network (Table 6.1.) and suggested to be involved in the control of cell motility and cell-cell adhesion.

Additionally, most of these genes have been reported to be mutated in different cancer types. For example, 8 of the examined 16 genes have been reported to be mutated in 100% of the analyzed ovarian tumor samples, 7 genes have been found to have mutations in 8% of the examined breast tumor samples and 4 genes have been shown to be mutated in 25-100% of the investigated intestinal tumor samples (Table 6.2.). On the other hand, all of these genes have been found to be differentially expressed in different cancer types. For example, *MGAT1* and *TPT1* have been reported to be upregulated in many cancer types including leukemia, brain, sarcoma and renal (kidney) cancers, whereas *FEN1*, *ARF1*, *CFL1* and *ARHGAP1* were found to be downregulated in sarcoma (Table 6.3.) supporting their potential roles in tumorigenesis.

Table 6.2. Putative Wnt/β-catenin target genes with detected mutations in different cancer types (adapted from Forbes *et al.*, 2011).

Gene	Tumor Sample	Sample Number	Samples with Mutations	Mutation Type
MGAT1	Ovarian	2	100%	2 missense
TINP1	Ovarian	1	100%	1 silent
	Breast	12	8%	1 missense
CALM3	Ovarian	1	100%	1 missense
Mortalin	Breast	12	8%	1 silent
	Intestinal	4	25%	1 missense
ENAH	Ovarian	1	100%	1 silent
	Breast	12	8%	1 missense
	Skin	1	100%	1 missense
	Intestinal	1	100%	1 missense

ARHGAP1	Ovary	1	100%	1 missense
	Skin	6	17%	1 missense
	Intestinal	4	25%	1 silent
CNN3	Intestinal	1	100%	1 missense
DEF8	Brain	44	2%	1 missense
IDE	Ovarian	1	100%	1 missense
	Breast	12	8%	1 missense
	Brain	44	2%	1 missense
FEN1	Ovarian	59	2%	1 missense
ARF1	Breast	12	8%	1 missense
PTPRF	Ovarian	4	100%	3 missense and 1 nonsense
	Breast	13	8%	1 silent
	Brain	45	2%	1 nonsense
	Kidney	101	1%	1 missense
	Liver	1	100%	1 missense
	Lung	11	9%	1 silent
	Skin	9	44%	2 missense and 1 nonsense
RAP1B	Breast	187	1%	2 missense
TPT1	-	-	-	-
HINT1	-	-	-	-
CFL1	-	-	-	-

Table 6.3. Putative Wnt/β-catenin target genes differentially expressed in different cancer types (adapted from Rhodes *et al.*, 2004). "+" indicates the upregulation and "-" denotes the downregulation of the gene in the analyzed tumor sample.

Gene	**Leukemia**	**Brain**	**Sarcoma**	**Lymphoma**	**Melanoma**	**Liver**	**Breast**	**Renal**	**Ovarian**	**Lung**
MGAT1	+	+	+	+	+			+		
TPT1	+	+	+			+	+	+	+	+
CALM3	+		+		+	+	-			
TINP1		+	+				-		+	+
ENAH		+	+		-	+				
FEN1			-				+		-	+
HINT1	-	+	+					+		
CNN3	+	+		+	+					-
DEF8	-			+	+	+	+		+	
IDE		+	+		+	-			+	
Mortalin		+	+	-				-		+
ARF1	+		-				+		+	+
CFL1			-				-		+	
PTPRF			+		-				+	
RAP1B			+				+		-	
ARHGAP1			-			+	+			

Having the purpose to elucidate the potential roles of these genes in the Wnt/β-catenin signaling pathway and tumorigenesis, several additional *in vitro* studies with some of these genes are being presently performed in our laboratory, as well. For instance, *Mena*, which was found to be upregulated upon β-catenin induction in HCC cell line, has been shown to be regulated by the Wnt/β-catenin pathway (Najafov *et al.*, 2012). On the other hand, *CNN3*, which was found to be downregulated upon β-catenin induction, has been shown to interact with ring finger protein 10 (RNF10) and RAS p21 protein activator (RASA1) proteins, which are involved in the regulation of cellular proliferation and differentiation and give clues about the potential role of *CNN3* in tumorigenesis (Dindar *et al.*, unpublished data).

Nevertheless, the roles of these genes in cancer and the molecular mechanisms behind their functions are still largely undefined pointing these genes as important subjects for cancer studies. Therefore, this study is of great importance, since the analyzed 16 genes are characterized as putative tumor promoting or tumor suppressing players of the cancer network in this study for the first time. Having the potential to suppress or further enhance *in vivo* tumor and metastasis formation is strong evidence supporting the roles of these genes in cancer progression.

However, in order to confirm these data, overexpression studies of the same genes may be performed in the same eyeful and sensitized backgrounds. Especially, for the genes which are suggested to have oncogenic functions according to the results in the eyeful and sensitized backgrounds, it would be a supportive evidence to observe promoted tumor or metastasis formations in the same fly cancer models upon the overexpression of these genes.

On the other hand, in the eyeful and sensitized flies activated Notch signaling via Delta overexpression is used as tumor-inducing background where genes affecting tumor and metastasis formation prevalences may be screened. Therefore, it is still unclear whether the examined 16 genes affect tumor and metastasis formation prevalences in the eyeful and sensitized backgrounds independently or by interacting with the Notch signaling pathway. In order to clarify this point, downregulation or overexpression srudies should be performed in wildtype flies by ignoring the activation of the Notch signaling

pathway via Delta overexpression. According to these results, genes which are able to increase or decrease the eye size of flies or to promote novel tumor or metastasis formations upon their downregulation or overexpression in wildtype flies, may be identified as independent players that may regulate cell cycle progression, cell proliferation or programmed cell death mechanisms. In contrast to these, genes whose downregulations or overexpressions do not affect the eye size or induce tumorigenesis in wildtype flies in the absence of overactivated Notch signaling may be considered as interacting partners of Notch pathway which may play key roles in tumorigenesis in collaboration with this signaling pathway.

Furthermore, the exact functions of the examined 16 genes in cell proliferation and apoptosis mechanisms in the cells are still unclear. It would be interesting to examine the effects of these genes on cell proliferation and apoptosis by analyzing the levels of proliferation markers such as phospho-HistoneH3 and apoptosis markers such as caspase-3 in the eye tissues of eyeful, sensitized and wildtype flies upon downregulation of the 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.

Apart from this, these 16 genes are identified as novel potential Wnt/β-catenin target genes, since they were found to be differentially expressed upon β-catenin induction mimicking overactivated Wnt signaling. In addition to their potential tumorigenic functions, 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 network in which they are involved in. For this purpose, identification of their interaction partners may be a first step for the determination of their exact action mechanisms, both as being a novel target of the Wnt/β-catenin pathway and playing an important role in tumorigenesis. Furthermore, a possible cross-talk between the Wnt/β-catenin signaling pathway and other signaling cascades via these putative target genes may be elucidated eventually.

With all these aspects, these examined 16 putative Wnt/β-catenin target genes with suggested tumor suppresor or oncogenic activities may be considered as novel subjects for cancer studies. By elucidating the possible roles of these genes in tumor 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 several cancer types. In addition to this, since these genes have not been previously found to be as a part of the Wnt/β-catenin signaling pathway, elucidation of their roles in the signaling network and identification of their interaction partners may provide general further knowledge about the Wnt/β-catenin signaling pathway, as well.

6.2. *Mena* as a Novel Candidate Gene in the Wnt-Notch Crosstalk

Mena is an actin-regulatory protein which belongs to ENA/VASP protein family. Members of this protein family are localized at the tips of protruding lamellipodia, filopodia and adhesion foci; and they are involved in control of cell motility and cell-cell adhesion, which are important subjects for development of metastatic potential. As many actin-associated proteins play important roles in carcinogenesis of various types of cancers, it has been shown that human *MENA* is overexpressed in ~75% of primary breast cancers (Di Modugno *et al.*, 2004).

In previous studies performed in our laboratory, *MENA* was found to be upregulated upon β-catenin induction mimicking overactivated Wnt signaling in the HCC cell line. Subsequent studies in our laboratory, supported the role of *MENA* as a transcriptional target of the Wnt/β-catenin pathway. Using luciferase reporter assays, it has been confirmed that the putative TCF4-binding elements conserved among mammalian homologues of *MENA* are indeed functional and are regulated by the Wnt/β-catenin pathway. Also, GSK3β inhibition by lithium treatment showed that *MENA* mRNA levels increase concurrently with the cytosolic accumulation and nuclear translocalization of β-catenin. Chromatin immunoprecipitation assays using Huh7 and HEK293 cells, as well as adult mouse brain and liver tissues showed that β-catenin directly interacts with the *MENA* promoter. Additionally, it has been shown that *MENA* mRNA levels are strongly upregulated in several human solid brain tumors (2- to 6-fold increase).

In consistence with its role as a Wnt/β-catenin pathway target, *MENA* has also been suggested to be involved in tumorigenesis. For instance, *MENA* is overexpressed in ~75%

of breast cancers and the Wnt/β-catenin pathway is overactivated in many breast cancer cell lines and tumors. Moreover, recently, *MENA* was found to be overexpressed in colon cancer and Wnt/β-catenin pathway overactivation is one of the major causes of colon carcinomas.

In this study, we showed that *MENA* plays a role in repressing Notch-mediated tumorigenesis in *Drosophila* eye cancer models. This is in contrast to the previously reported role of *MENA* as a tumorigenesis-promoting protein, as *MENA* has been found to be overexpressed in ~75% of primary breast cancers and play a role in activation of MAPK and Akt signalling. Therefore, our data put *MENA* at a critical point of crosstalk between Wnt/β-catenin and Notch pathways by providing a novel example of a Wnt target that represses Notch signalling (Najafov *et al.*, 2012).

Since Notch mediated tumor and metastasis formations in eyeful and sensitized cancer models were found to be promoted upon downregulation of *MENA*, and since *MENA* was found to be a Wnt/β-catenin pathway target, this gene may function in a possible Wnt-Notch crosstalk where Wnt signaling may present an inhibitory effect on Notch signaling via *MENA*. In order to question this possibility, we downregulated *MENA* in HEK293FT cells *in vitro* and examined the expression profiles of several Notch target genes upon this downregulation. Consequently, Notch targets were found to be upregulated upon *MENA* downregulation in the analyzed cell line supporting the inhibitory effect of *MENA* on Notch signaling.

Like Wnt signaling, aberrrantly activated Notch signaling has been observed during the carcinogenesis of many human cancers, such as pancreatic cancer, breast cancer, prostate cancer, liver cancer, lung cancer and ovarian cancer. In addition, overexpression of Notch signaling was found to be associated with poor prognosis or poor response to treatment of some solid tumors such as breast tumor and prostate cancer. Thus, Notch signaling has been proposed as an important target for cancer therapy (Qiao *et al*., 2009).

The Wnt and Notch signalling pathways play important roles in development and tumourigenesis and the crosstalk between these two pathways including genetic interactions in *Drosophila*, the physical binding of Notch to β-catenin or their association

to common cofactors have been described. For instance, in mammalian cells, GSK3β directly phosphorylates the Notch protein thus modulating its transcriptional activity. Moreover, β-catenin activates Jagged1 transcription, thus leading to Notch activation during murine hair follicle differentiation (Rodilla *et al.*, 2009).

In tumorigenesis there are many examples where Wnt and Notch pathways collaborate with each other by enhancing or suppressing their functions via different molecules. However, the exact mechanisms of these interactions and the contributions of these crosstalks to tumorigenesis are still largely undefined. In order to reveal novel molecular partners of the protein Mena, yeast two hybrid analysis would be an essential experimental step to perform. Eventually, the association of the identified novel interaction partners with Wnt and Notch signaling pathways may be investigated in order to enlighten the molecular network behind the proposed Wnt-Notch crosstalk. Elucidating the molecular mechanism of these collaborations and identifying the interaction partners of both signaling pathways may provide general further knowledge about these signaling pathways and their contributions to tumor and metastasis formations in different cancer types. In this manner, *Mena*, which is giving clues about a novel Wnt-Notch crosstalk, might be pointed as a novel target for future studies.

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