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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

The inverse relation between carcinogenesis and regeneration: a knockdown study of Glipr1 and Mmpb in *Schmidtea mediterranea*

Promotor :
Prof. dr. Karen SMEETS

Copromotor :
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Erwin Leysen
Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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Foreword

As a first part of this work, I would like to take the time to thank some of the people who helped and supported me in completing this Master thesis.

Not only was working in the lab of the department of Animal Science for nine months a very enjoyable experience, both the practical work and the writing of this work has also taught me a great deal, on a professional level as well as on a personal level. I would like to thank prof. dr. Karen Smeets for granting me the opportunity to do my internship as part of her research group, but also for her guidance and patience as my promoter.

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Last but definitely not least I would like to thank my mom and dad for the opportunity to spend so many years of my life on my education. I am forever grateful for your support.

Thank you.

List of abbreviations

2D-DiGe	2D-difference gel electrophoresis
APC	Adenomatous polyposis
BCL-2	B-cell CLL/lymphoma 2
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
Cd	Cadmium
$CdCl_2$	Cadmium chloride
CTNNB1	Beta-catenin gene
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
ERK	Extracellular regulated kinase
GLIPR1	Glioma pathogenesis related protein 1
H_2O_2	Hydrogen peroxide
H&E	Hematoxylin and eosin
HCl	Hydrochloric acid
HIF-1	Hypoxia-inducible factor 1
JNK	C-jun N-terminal kinase
KCl	Potassium chloride
MAPK	Mitogen-activated protein kinase
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MMP19	Matrix metalloproteinase 19
MMPB	Matrix metalloproteinase B
MMS	Methyl methanesulfonate
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NOTUM	Notum pectinacetylesterase homolog
NRF2	Nuclear factor, erythroid 2-like 2
P16	Cyclin-dependent kinase inhibitor 2A
P53	Tumor protein P53
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-triton
RB	Retinoblastoma 1
Ref-1	Redox effector factor 1
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
S. mediterranea	*Schmidtea mediterranea*
Trx	Thioredoxin
WNT	Wingless-type MMTV integration site family

Summary

Carcinogenesis occurs less often in highly regenerative tissues and organisms, for example in the liver. Another example is an invertebrate; the planarian *Schmidtea mediterranea* (*S. mediterranea*), of which it is known that it possesses a vast regenerative capacity due to a large population of pluripotent stem cells called neoblasts that enables the organism to regenerate its entire body, including its brain, in only five days. *S. mediterranea* is able to circumvent tumor formation, even after prolonged exposure to genotoxic and non-genotoxic carcinogens, although the specific mechanism of this process is unknown. However, it was observed that the dubious carcinogenic compound cadmium initiates a hyperproliferative response in exposed animals, a response that could be induced in order to circumvent carcinogenesis. In order to investigate the molecular mechanism behind the circumvention of carcinogenesis, a non-hypothesis driven screening study was performed at the moment of the hyperproliferative response. As a result of the screening study, specific proteins that showed higher quantities during the hyperproliferative response were identified and were hypothesized to be involved in the circumvention of carcinogenesis.

Two proteins that were among the proteins with the largest quantitative deviations were investigated first, namely glioma pathogenesis related protein 1 (Glipr1) and matrix metalloproteinase B (Mmpb). A technique called RNA interference was used as a mean to induce a specific gene knockdown of these proteins, after which *S. mediterranea* were cut in three parts to induce regeneration, and subsequently exposed to carcinogenic compounds such as cadmium (10μM) or methyl methanesulfonate (50μM).

A gene knockdown of both genes led to phenotypic changes, such as local tumor-like outgrowths, in *S. mediterranea*. In order to classify these observed outgrowths as tumors, further characterization by means of light microscopy and mitotic analysis was required. The light microscopic analysis showed that the outgrowths consisted of tissue, and thus that they were not merely the result of for example local fluid build-up or blister-like lesions. The tissues in the outgrowths were mostly hypertrophic and hyperplastic gastrointestinal cells, and tissue of the neuronal line. Furthermore, the mitotic analysis showed that there are dividing cells present on the site of the outgrowth, although a quantitative measure of the dividing cells has not yet been performed.

To conclude, it can be suggested that Glipr1 and Mmpb exert tumor-suppressing functions in *S. mediterranea*. However, more research is needed in order to further investigate the characteristics of the outgrowths, for instance by using electron microscopy. Since the investigated genes play a role in the hyperproliferative response of *S. mediterranea* as a result of cadmium exposure, it is likely that this hyperproliferative response is a way of circumventing carcinogenesis in *S. mediterranea*. A knockdown of only one gene can have a large effect on regenerating animals; hence it is clear that the regulation of the circumvention of carcinogenesis is a very delicate process. However, more research is needed in order to investigate the important role of the microenvironment on this process and to translate this information to other highly regenerative tissues and organisms.

Samenvatting

Carcinogenese komt minder vaak voor in sterk regenererende weefsels en organismen, zoals bijvoorbeeld in de lever. Een ander voorbeeld is de ongewervelde platworm *Schmidtea mediterranea* (*S. mediterranea*), waarvan geweten is dat het een enorme regeneratieve capaciteit bezit als gevolg van een omvangrijke populatie van pluripotente stam cellen die ervoor zorgen dat dit organisme zijn gehele lichaam, inclusief zijn hoofd, kan regenereren in slechts vijf dagen. *S. mediterranea* kan tumorvorming ontwijken, zelfs na een langdurige blootstelling aan genotoxische en niet-genotoxische carcinogenen. Om het mechanisme van de ontwijking van carcinogenese te onderzoeken, maar ook om de mogelijke link met regeneratie te kunnen aantonen, werd een open screening studie uitgevoerd waarbij intacte en regenererende *S. mediterranea* werden blootgesteld aan de carcinogene stof cadmium. Specifieke proteïnen met veranderde hoeveelheden als gevolg van de aanwezigheid van cadmium werden geïdentificeerd. De hypothese is dat het net deze proteïnen zijn die een rol spelen in de ontwijking van carcinogenese.

Twee proteïnen die deel uitmaakten van de proteïnen die de grootste kwantitatieve afwijking vertoonden, werden eerst onderzocht. Deze proteïnen waren glioma pathogenese-gerelateerde proteïne 1 (Glipr1) en matrix metalloproteïnase B (Mmpb). Een techniek die RNA interferentie wordt genoemd werd gebruikt om een specifieke gen-knockdown van deze proteïnen te bekomen, waarna de dieren in drie delen werden gesneden om regeneratie uit te lokken, om daarna blootgesteld te worden aan carcinogene stoffen zoals cadmium (10µM) en methyl methaansulfonaat (50µM).

Een gen-knockdown van de onderzochte genen leidde tot fenotypische veranderingen zoals lokale tumor-achtige uitgroeisels in *S. mediterranea*. Om deze geobserveerde uitgroeisels te kunnen classificeren als tumoren was een verdere karakterisering nodig door middel van licht microscopie en een mitotische analyse. Licht microscopie toonde aan dat de uitgroeisels uit weefsel bestonden, en dat zij dus niet slechts het gevolg waren van een lokale vochtretentie of blaar-achtige laesies. Daarbovenop toonde de mitotische analyse dat de cellen op de plek van het uitgroeisel delende cellen waren, hoewel er nog geen kwantitatieve analyse is uitgevoerd.

Om tot een conclusie te komen kan er gesuggereerd worden dat Glipr1 en Mmpb een rol spelen in de ontwijking van carcinogenese door *S. mediterranea*. Het is echter nodig om meer onderzoek te verrichten om de karakteristieken van de uitgroeisels verder te kunnen bepalen, bijvoorbeeld door middel van elektronenmicroscopie. Aangezien de onderzochte genen een rol lijken te spelen in de hyperproliferatieve respons van *S. mediterranea* ten gevolge van cadmiumblootstelling, is het aannemelijk dat deze hyperproliferatie een manier is om carcinogenese te ontwijken. Aangezien een knockdown van slechts één gen een groot effect kan hebben op wat er gebeurt met *S. mediterranea*, is het duidelijk dat de regulatie van de ontwijking van carcinogenese een zeer delicaat proces is. Het is dus ook duidelijk dat meer onderzoek onontbeerlijk is om meer informatie te vergaren omtrent het verband tussen regeneratie en carcinogenese.

1. Introduction

1.1 Carcinogenesis

Cancer is defined as the uncontrolled division of mutated cells that leads to a malignant tumor. In the process of cancer formation (carcinogenesis), the control of cell proliferation, differentiation, migration and programmed cell death (apoptosis) is lost. Because of this, cells constantly divide, while in normal tissues the cell cycle is strictly regulated in order to maintain homeostasis [1,2]. As one of the main causes of death worldwide, with 8.2 million cancer deaths worldwide in 2012 alone, cancer is one of the most researched diseases, estimated to have cost over 14 billion euros in 2004 [3,4]. However, there is still need for more research, and especially for modern research methods.

Regeneration has been proposed as a possible source of cancer, since the cancer formation can be derived from an incomplete and/or impaired process of regeneration [5]. On the other hand, it is known that tissues and organisms with great regenerative potential, such as the human liver and the planarian *Schmidtea mediterranea* (*S. mediterranea*), show a relatively high resistance to cancer formation by preventing, and even correcting, growth abnormalities [6,7]. However, the underlying mechanisms remain mainly unknown [6]. By studying these mechanisms, both on a cellular and a molecular level, involved in the control of cell growth in both regeneration and carcinogenesis, more knowledge may be gained about possible key regulators of carcinogenesis.

1.1.1 Three stages of carcinogenesis

The carcinogenic process is usually divided into three well-characterized stages, namely initiation, promotion and progression. During the initiation stage, an interaction between a carcinogen, or its metabolite, and nucleic acids occurs, which subsequently leads to mutations or methylations in tumor suppressor genes, or in oncogenes, or, in a rare occasion, these alterations develop spontaneously. As a result of this interaction, the genotype of the affected stem cell is irreversibly changed, inducing its immortality. The initiation stage can take place even after a short exposure to a carcinogen, which implies that the dose of the carcinogen is relevant [8,9].

The promotion stage is characterized by the clonal expansion of initiated cells, which produces a relatively large population of cells that are at risk of further genetic changes and subsequent malignant conversion, since the amount of acquired mutations is directly proportional to the stem cell division rate. Unlike the initiation stage, a prolonged exposure to a carcinogen, referred to as a tumor promoter, is required for promotion to occur, which implies that tumor promotion may be a reversible process to a certain extent. Thus, the dose of the tumor promoter is usually of lesser significance compared to the frequency of repeated administrations. Chemicals involved in both tumor initiation and tumor promotion are known as complete carcinogens [8-11].

As preneoplastic cells transform into cells that express a malignant phenotype in a process called malignant conversion. As a result of malignant conversion more aggressive

characteristics are acquired over time, which is referred to as tumor progression. The malignant phenotype is characterized by the propensity for uncontrolled growth and genomic instability, either at the nucleotide level by insertions or deletions of a few nucleotides, or at the chromosome level by gains or losses of whole chromosomes or portions thereof. Tumor cells may also become able to secrete proteases, which can lead to metastasis, an invasion of tissue in the proximity of the primary tumor location [8-11]. However, it should not be assumed that solely these three distinct stages can be defined since each stage can be subdivided into many substages, in which for example the activation of enzymes, alterations of the metabolism, and the prevention of cell differentiation and cell communication play an important role [8-11].

1.1.2 Factors affecting carcinogenesis

The aforementioned three stages of carcinogenesis are influenced by several factors, for instance age, dietary non-genotoxic components, environmental factors such as physical and chemical mutagens (e.g. asbestos, UV irradiation, cigarette smoke, and heterocyclic amines), infections by certain pathogens such as bacteria or viruses (e.g. human papilloma viruses, Epstein-Barr virus, *Salmonella typhi*, and *Chlamydia pneumonia*), oxidative stress, as well as germline mutations in cancer-related genes. These factors may induce either genetic changes or epigenetic changes, or both, which may lead to the initiation and/or promotion of tumors, as described before. Other micro-environmental factors, such as the presence of growth factors and pro-angiogenic factors, aid in the development of tumors. It is important to gain insight in the effect of genetic alterations of (proto)-oncogenes and tumor suppressor genes, the consequence of the oxidative misbalance, and exposure to chemical carcinogenic compounds in order to use this insight for specific treatment in the future. Comparing these mechanisms in tissues and organisms that are tumor-sensitive with those can circumvent tumor formation will gain more knowledge about these processes [9,12].

1.1.2.1 (Proto)-oncogenes and tumor suppressor genes

Most genetic changes that have an important impact in the multi-stage process of carcinogenesis occur in certain groups of genes, called oncogenes and tumor suppressor genes, although other gene alteration can also be detrimental. When critical functions of a cell are altered, the cell receives signals to undergo apoptosis. However, activated oncogenes are capable of not only withholding the cells with altered functions from undergoing apoptosis, but even of inducing a signal for cell proliferation, leading to the expansion of genetically altered cells, which may lead to even more genetic instability and, eventually, a tumor. Contrary to oncogenes, tumor suppressor genes usually protect the cell from becoming cancerous, for example by repairing DNA damage or by inhibiting cell division in the presence of DNA damage. When genetic changes diminish the effectiveness of tumor suppressor genes to counteract the presence of DNA damage, said DNA damage may not be repaired, and cell division will continue, leading to a larger population of genetically instable cells [11,13,14].

The protein products of proto-oncogenes are usually involved in either the regulation of cell differentiation or cell growth, in the execution of mitogenic signals or in signal transduction. As a result of modifications of its original function, proto-oncogenes, which are normal genes, become oncogenes, which are tumor-inducing genes [14]. These modifications can be induced by gain-of-function mutations within the proto-oncogene itself, or within its promoter region, hereby increasing the activity or amount of the resultant protein encoded by the oncogene, or changing the protein structure [13-15]. In many cases, proto-oncogenes are activated by one of two major mechanisms, namely point mutations of specific regions of the genes that belong to the *RAS* gene family, or amplification of the chromosomal segments containing members of specific multigene families, for example the jun proto-oncogene family (*JUN*) [11,16]. However, it is known that c-Ha-ras plays an important role in the control of regeneration of the liver and both a loss and an upregulation of the gene may cause liver cancer, while phosphorylated Junb proteins are necessary for tissue regeneration in both larval and adult zebrafish [17,18]. Both mechanisms lead to an overexpression of the affected genes. Overexpression of some oncogenes may also occur as a result of translocations by which the genes become juxtaposed to a powerful promoter. An example is the translocation of the B-cell CLL/lymphoma 2 (*BCL-2*) gene so it becomes juxtaposed to the immunoglobulin lambda light chain gene promoter, which is a process that leads to chronic lymphocytic leukemia [11,16,19].

On the other hand, the proteins encoded by tumor suppressor genes usually have a repressive effect on the regulation of the cell cycle and/or promote apoptosis. More specifically, tumor suppressor genes exert their effect on the cell cycle either by directly repressing genes that are necessary for its continuity, or by pairing the cell cycle with DNA damage by which cells with DNA damage will no longer divide. When the DNA damage cannot be repaired, the apoptotic process, which is also strictly regulated by tumor suppressor genes, is initiated. A loss of function of tumor suppressor genes that are involved in cell adhesion promotes metastasis [6,12]. Examples of tumor suppressor genes include tumor protein P53 (*P53*), cyclin-dependent kinase inhibitor 2A (*P16*) and the retinoblastoma 1 (*RB*) gene. The loss is these genes mentioned above are proven to contribute to carcinogenesis in a direct way. In this project two tumor suppressor genes will be elucidated further since their functions will be studied extensively, namely glioma pathogenesis-related protein 1 (*GLIPR1*) and matrix metalloproteinase 19 (*MMP19*). Both genes emerged as possible important genes in the circumvention of carcinogenesis as a result of a non-hypothesis driven study in which proteins were discovered that were altered significantly as the result of the exposure to the carcinogen cadmium (Cd). *GLIPR1* has a function in the induction of apoptosis and in tumor suppression, while *MMPB19* is known to play a role in cell migration and development [20-22]. Unlike proto-oncogenes, loss-of-function mutations reduce the function of tumor suppressor genes and said mutations must affect both alleles before an effect is eminent, which is referred to as the two-hit hypothesis [15,23]. It is postulated that the loss of function of tumor suppressor genes may be more eminent than the activation of proto-oncogenes/oncogenes in the

formation of many kinds of human cancer [24]. However, usually both dysfunctions occur in carcinogenesis [25].

1.1.2.2 Oxidative balance

Aerobic organisms, such as humans, are dependent on oxygen for the main part of its energy production. Oxidative reactions occur constantly in the body, mainly in the mitochondria, producing free radicals usually referred to as reactive oxygen species (ROS), as a byproduct. However, since there is a constant production of ROS and thus a constant thread of damage as a result of the presence of ROS, extensive antioxidative mechanisms have developed in aerobes. When the balance between the pro-oxidants (ROS) and the antioxidants is altered in favor of ROS, due to either a redundancy of ROS or a scarcity of antioxidants or a combination of both, oxidative stress occurs. For example, ROS levels can be increased during endogenic processes such as chronic inflammation, as well as by exogenous carcinogens [26-27]. The free radical theory postulates that oxidative stress can cause extensive damage to a wide range of macromolecules, such as lipids, proteins, and nucleic acids. Interactions of ROS with DNA oligonucleotides can cause DNA cross-links, single- and double-strand DNA breaks, as well as deoxyribose, purine or pyrimidine modifications, which may all lead to mutations. Said mutations may initiate tumor formation when they occur in (proto)-oncogenes or tumor suppressor genes, as discussed earlier [26,28]. Interestingly, ROS are required for several physiological processes that are also eminent for carcinogenesis, such as the induction of proliferation, apoptosis and differentiation, as well as stem cell maintenance. Proliferation is induced by ROS by stimulation of the mitogen-activated protein kinase/extracellular regulated kinases (*Mapk*/*Erk*) pathway and subsequent activation of transcription factors such as nuclear factor, erythroid 2-like 2 (Nrf2) and hypoxia-inducible factor 1 (Hif-1), as well as by activation of the thioredoxin/redox effector factor 1 (Trx/Ref-1) complex. These transcription factors have prominent roles in the processes of regeneration and carcinogenesis. Activation of the c-jun N-terminal kinase gene family (*JNK*), an inducer of apoptosis, is dependent of sufficiently high ROS levels, and it is JNK that phosphorylates proto-oncogene JUN, a gene that is responsible for both regeneration in zebrafish, as well as for carcinogenesis [18,26-28].

In carcinogenesis, as well as in regeneration, it is the stem cell population that proliferates and either forms tumors or repairs damage, respectively. In the process of stem cells maintenance, the oxidative balance is crucial. In general, a low base level of ROS supports stem cell quiescence, while a slight increase in the ROS level induces stem cell proliferation [26-28]. On the other hand, ROS has a direct effect on the induction of apoptosis by acting as upstream signaling molecules, but ROS can also indirectly induce apoptosis when causing irreparable DNA damage, which activates tumor suppressor genes' ability to initiate cell death. Anti-apoptotic oncogenes such as *BCL-2* also play a role in the ROS balance by preventing the accumulation of ROS. As a consequence of the aforementioned factors, oxidative stress may be a factor in the process of carcinogenesis [26-29].

1.1.2.3 Carcinogenic compounds

Carcinogens are also an important factor to consider in carcinogenesis. Carcinogens include chemical structures and their metabolites, as well as radionuclides and radiation that can directly lead to the development of cancer by influencing factors that affect carcinogenesis, such as oncogenes and tumor suppressor genes, and the oxidative balance. One of the proposed reasons why the cancer incidence has been on a rise is the daily exposure to carcinogens as a result of pollution and the use of cleaning products, as well as for example preservatives and pesticides in and on food [30,31]. The effect of carcinogenic compounds can be categorized as genotoxic or non-genotoxic. Genotoxic carcinogens cause direct damage to the DNA, which can activate proto-oncogenes or silence tumor suppressor genes, whereas non-genotoxic carcinogens do not react directly with the DNA, but for example cause epigenetic changes or influence the oxidative balance. In this work, both genotoxic and non-genotoxic carcinogens are used since it is interesting to investigate the effect of both classes of substances on the development of tumors [32,33]. Genotoxic carcinogens usually act as tumor initiators that even exhibit proportional responses at low doses, while non-genotoxic carcinogens are assumed to play a role as tumor promoters that exhibit threshold tumor dose-responses. Examples of genotoxic agents include asbestos, arsenic, formaldehyde, benzene and tobacco, among many others [34-38]. Methyl methanesulfonate (MMS) is a genotoxic carcinogen that is frequently used in our laboratory. MMS functions through DNA alkylation. As a result of exposure to MMS, methylation occurs to the adenine-guanine base pair, causing replication blocks and DNA mismatching, subsequently leading to a hindrance of the progression of the replication fork. MMS is actually used in the treatment of cancer [37]. On the other hand, examples of non-genotoxic compounds include dioxins and ethanol. Sometimes it is hard to clearly distinguish both groups of carcinogens since there are various secondary effects [37-41]. Many metals are also characterized as genotoxic carcinogens, such as chromium hexavalent compounds and nickel [41-43]. One of the primary mechanisms of metal-induced carcinogenicity is ROS-induced DNA damage [26].

However, next to genotoxic and non-genotoxic compounds, there are also compounds that have not been characterized as either genotoxic or non-genotoxic, since it is not clear yet. One of these is Cd, which is a carcinogen that is frequently used in our research group as a non-essential metal that is toxic even in low concentrations. Although there is more DNA damage present after exposure to Cd *in vivo*, making Cd a weak genotoxic carcinogen, this effect is not consistently seen *in vitro*. It is suggested that the DNA damage observed upon Cd exposure is either indirect, or Cd inhibits DNA repair, which is why Cd could work synergistically with several mutagens and carcinogens [44,45]. Cd induces oxidative stress by a disturbance in the mitochondrial function, as well as by an inhibition of the antioxidative defense system, which may cause indirect DNA damage [26,45]. Cellular effects of Cd exposure include DNA methylation and the inhibition of DNA repair mechanisms, thus the DNA damage caused by Cd-induced oxidative stress may not be repaired [44-46].

1.2 Regeneration

Regeneration is a biological process that naturally replaces extruded body parts, such as cells of the epidermis or of the epithelia of the gut, or for example red blood cells, in humans [47]. In mammals, another tissue that has a high regenerative capacity is the liver; in fact it is the only visceral organ that has sufficient capacity to regenerate. Even after a 75 percent reduction of the original liver mass, due to either chemical injury or surgical removal, the liver can regenerate to its full size. The liver is almost constantly exposed to many carcinogens and other poisonous substances because of its function, yet the tissue is not very prone to carcinogenesis, possibly because of its vast regenerative capacity [7,17]. One subtype of regeneration, epimorphic regeneration, restores damaged tissue (e.g. a wound) to its original state, without loss of function [48]. In several invertebrate organisms, such as the planarians, epimorphic regeneration can be used to restore an entire organism from only a limited fragment of tissue [6]. However, in humans, epimorphic regeneration is limited in many tissues and organ systems, such as most prominently known cells of the neuronal lineage, which is why paralysis caused by damage to the spinal cord is usually irreversible [49]. The process of regeneration, as well as its regulation, is well understood, as will be described later. However, by comparing the process of regeneration with that of carcinogenesis, more insight in the latter process may be gained [48-49].

1.2.1 Process of regeneration

The process of epimorphic regeneration can be subdivided into several stages that are well coordinated and strictly regulated, as will be explained later. First of all, a trauma occurs, causing damage to tissue. In order to restore the damage, the regeneration process is initiated. The wound closure occurs by the contraction of muscle surrounding the wound site, while major vessels contract in order to terminate bleeding in humans; the latter process is missing in planarians because of the absence of a circulatory system. Subsequently, filaments are reorganized and a wound epithelium is formed. Fourthly, dedifferentiation and proliferation of adjacent stem cells is induced, along with proliferation of more distant stem cells that migrate towards the site of damage [47,48]. Formerly quiescent stem cells proliferate as a result of stimulation by serine proteases such as thrombin [48]. An infiltration of inflammatory cells occurs as well at this stage, causing an acute inflammation, which is also a process that may not occur in planarians although the immune system and its potential role in regeneration is mostly unknown. A regeneration blastema is formed as a result of the presence of proliferating stem cells, which is the colorless region from which regeneration is induced. These stem cells will eventually differentiate towards the intended tissue and will replace the lost cells in the damaged tissue, or the entire lost body part if required [47,49,50,51]. After restoring the damaged tissue to its original state, the process is terminated [48]. However, contrary to planarians that can regenerate their entire body, in humans this process does not take place in every tissue, for example damage to the spinal cord can not be repaired since the neuronal stem cells are not prone to start migrating and proliferating. Stem cell therapy could be used to

treat paraplegia, although more research is needed, especially on the relationship between regeneration and carcinogenesis in order to support regenerative medicine [49,50]

1.2.2 Regulation of regeneration

One important aspect of the regulation of regeneration is the delicate balance between three cellular processes, namely proliferation, differentiation, and apoptosis. A network of systems, including signal transduction pathways and ROS, regulate these cellular processes [47,52]. For example, the β-catenin (*CTNNB1*) gene is required for correct polarization by promoting tail structures and inhibiting head structures, while the proto-oncogene family known as the wingless-type MMTV integration site family (*WNT)* is important for cell fate specification, as well as for cell proliferation and migration, and patterning [53,54]. These two genes together are usually referred to as the *WNT*/*CTNNB1* pathway, and it is known that ROS are important regulators hereof. *CTNNB1* is also inhibited by the tumor suppressor genes adenomatous polyposis coli (*APC*) and notum pectinacetylesterase homolog (*NOTUM*), among others. The latter is also a known suppressor of *WNT* signaling [53,54]. Other regulating factors of mammalian regeneration include the presence of (angiogenic) growth factors, which induces and supports proliferation. When the regulatory signals are absent or altered, uncontrolled cell division can occur, which may lead to malignancies [47,49,53]. Voltage is also a regulating factor in the regeneration of planarians, with a membrane depolarization leading to head formation, even in posterior-facing wounds [54]. The distal extracellular matrix, which is important for the stem cell niche, is reorganized by matrix metalloproteinases such as MMP19, which is a gene that is further examined in this project after being identified by the non-hypothesis driven study as a gene that may be important in the circumvention of carcinogenesis by *S. mediterranea* [19,55]. It is also known that a difference in microenvironment in different tissues influences the outcome of the regenerating tissue, not only whether it regenerates into tissue of another lineage, but also whether inflammation and scarring occurs [56,57].

1.2.3 Regeneration and carcinogenesis

Although one is a physiological process, and the other is a malignancy, there are several similarities between regeneration and carcinogenesis. In general, the processes of carcinogenesis and regeneration and also their respective regulation are similar, but the outcome is different. Regeneration is strictly regulated and terminated, while carcinogenesis typically involves uncontrolled proliferation and the acquisition of invasive properties [25,49].

For both regeneration and carcinogenesis, four cellular processes are vital, namely proliferation, migration, apoptosis and differentiation [47,49,58]. However, the major difference between both is the absence of control over these processes in carcinogenesis, caused by numerous factors such as gain-of-function mutations in oncogenes, loss-of-function mutations in tumor suppressor genes, and possibly high ROS levels as may be the result of chronic inflammation, as described earlier, which are in fact factors that all help regulate the cell cycle in regeneration. Tumor suppressor genes such as *APC* and *NOTUM*

regulate regeneration, while carcinogenesis is only possible due to a loss of tumor suppressor function [9,59]. Proto-oncogenes, which code for some growth factors, are required for the processes of both regeneration and carcinogenesis while also ensuring nutrient and oxygen delivery, as well as waste and carbon dioxide disposal, through the induction of angiogenesis [23,58]. As a consequence, the initial gene expression profiles of the growth factors in both regeneration and carcinogenesis are highly comparable. However, when a gain-of-function mutation occurs to these proto-oncogenes, the adjacent cells will overproduce growth factors under influence of the oncogene, which will induce an overproliferation of the stem cells and a misbalance in the number of proliferating and apoptotic cells. Consequently, a tumor may form. Furthermore, the lack of a termination process leads to constantly dividing cells that may form a tumor, while regeneration is a strictly coordinated process with a clear cessation [25,47,49].

The ability to evade tumor formation is clearly seen in tissues and organisms with extensive regeneration capacity, such as the human liver and *S. mediterranea*, respectively, which is another reason to believe there is a link between both processes. It is even suggested that the regulation of regeneration may in fact have a positive effect on the incidence and extent of carcinogenesis. No tumors were detected in *S. mediterranea* after prolonged exposure to known genotoxic carcinogens such as MMS or 4 nitroquinoline-1-oxide, after exposure to known non-genotoxic carcinogens such as methapyrilene hydrochloride, cyclosporine A, chlorpromazine hydrochloride or sodium phenobarbital, as well as after exposure to the dubious compound cadmium [6,60,61]. In our research group, the next step was to use RNA interference in order to knock down tumor suppressor genes that are important in humans, such as P53, in *S. mediterranea* in order to investigate the dual role of this tumor suppressor gene in both regeneration and carcinogenesis [62]. A non-hypothesis driven study was performed in which the alterations of protein quantities was determined as a result of the exposure of *S. mediterranea* to cadmium chloride ($CdCl_2$). The proteins that were found and that were known possible tumor suppressors were examined further by means of RNA interference, being identified as Mmpb and Glipr1.

1.3 Planarians

1.3.1 *Schmidtea mediterranea* as a model organism

S. mediterranea is a freshwater flatworm that is a member of the phylum Plathylmenthes. *S. mediterranea* is capable of regenerating large portions of its body following injury, including its brain. No more than just a small fraction of an adult planarian is required in order to regenerate towards a complete adult organism in only seven days time, with a first proliferative peak occurring approximately six hours after cutting *S. mediterranea*, and the major proliferative peak 48-72 hours after cutting the animal. As a result of its immense regenerative potential *S. mediterranea* has been studied for over 200 years. For example aging, behavior, neuro- and organ regeneration, molecular differences during (adult) regeneration, the effect of ROS in regeneration, and neoblasts specialization were

all extensively studied [6,59,63-67]. As a result of the profound study of the organism, the *S. mediterranea* genome is fully sequenced and collected in a database, known as SmedGD [68]. However, using *S. mediterranea* to study carcinogenesis is a rather recent development. So far it is known that *S. mediterranea* do not form tumors after exposure to various carcinogens, although it has been shown that exposure to carcinogens does induce a proliferative response [6]. On the other hand, it is known that tumors have developed in other planarians, such as in *Dendrocoelum lacteum*, which is a planarian with a limited and strongly polarized regenerative capacity after exposure to chemicals. Interestingly, only the non-regenerative posterior develops tumors, while the regenerative anterior does not [50].

Benefits of using *S. mediterranea* include the ease and relative low cost of keeping the organism in culture. Exposure of *S. mediterranea* to several carcinogenic compounds is also relatively easy to perform since these carcinogenic compounds can simply be added to the aquatic culture medium, and overall handling of the model organism is quite straightforward. *S. mediterranea* can reproduce either sexually, or asexually through transverse fission. The benefit of using asexual *S. mediterranea* as a model organism is its genetic stability, which provides the possibility to cultivate thousands of flatworms that are genetically identical. Hence, an asexual strain of *S. mediterranea* is used in our research group. Furthermore, since planarians are invertebrates, there are less ethical regulations that are to be respected [64,69,70]. *S. mediterranea* is used in our research group mostly for toxicological studies, for example a toxicological assay has been developed to test the status of carcinogens.

1.3.2 Morphology of *Schmidtea mediterranea*

S. mediterranea are bilaterally symmetric organisms that are dorsoventrally flattened. Contrary to vertebrates, the planarian body lacks skeletal structures, as well as circulatory and respiratory systems. In fact, it may be because the absence of a respiratory system that *S. mediterranea* are dorsoventrally flattened since it facilitates oxygen exchange by diffusion [64,70,71]. All planarians are very basal triploblastic organisms, meaning that derivatives of all three germ layers ultimately form multiple organ systems in planarians, including a gastrointestinal tract and a central nervous system. The gastrointestinal tract consists of a mouth, distally located on the ventrally located pharynx, one branch of intestines in the anterior region, and two branches of intestines in the posterior region, lacking an anus. In other words, the *S. mediterranea* is a member of the tricladida. The gut branches are strongly diverticulated, which is required for dispersion of essential nutrients in absence of a circulatory system. Anterior cephalic ganglia, together with longitudinal central nerve cords that are connected by transverse commissures and surrounded by lateral branches, make up the central nervous system. Dorsal anterior photoreceptors are present, as well as auricles that may serve as sensory organs. Since *S. mediterranea* have less connective tissue, the organ structures seem less organized, as compared to mammals [62,64,70,71].

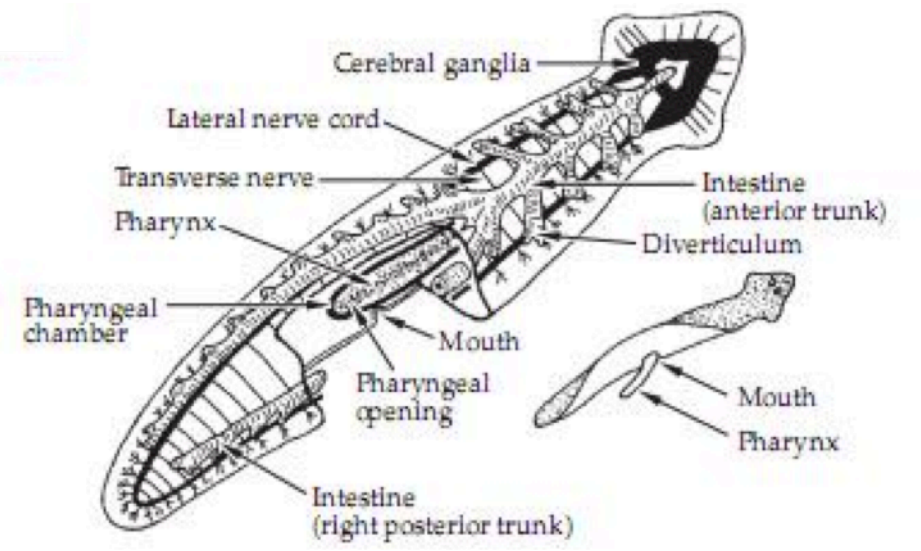

Figure 1: Morphology of *S. mediterranea*; central nervous system and gastrointestinal tract. Adapted from [69].

1.3.3 Neoblasts

As mentioned before, dividing stem cells are required for both carcinogenesis and regeneration to occur, since they are the driving force of cell proliferation, migration and differentiation, and thus these cells are the main target of gene therapies [9,51]. Adult planarians possess a large population of pluripotent stem cells called neoblasts, with up to approximately 30 percent of the planarian body consisting of these stem cells, providing these organisms with an enormous regeneration potential [69,72]. When lethally irradiating planarians so the stem cells are unable to proliferate, disabling regeneration, the regenerative capability can be restored through the transplantation of a single neoblasts from another planarian [73]. However, the population of neoblasts may not be homogenous, enabling different possible responses to varying injury types [74].

Neoblasts can be determined by their small size, round shape, little cytoplasm and relatively large nucleus, as well as their highly undifferentiated appearance [69,75-77]. However, the pharyngeal region and the region anterior of the eyes have no stem cells present, hence in these areas it would be expected to have a higher prevalence of tumor formation, since this phenomenon was also seen in *Dendrocoelum lacteum*, although the area without actively dividing stem cells is much smaller in *S. mediterranea* [77]. *In vivo* studies of the neoblasts of *S. mediterranea* are easily performed since the neoblasts are the only mitotically active cells in adult animals. Since exposure to carcinogens, and RNA interference (RNAi) through microinjections with double-stranded RNA, have a direct effect on the neoblasts population; phenotypic changes can be directly correlated to stem cell responses [69,76,77].

1.4 Objectives

Since the exposure of *S. mediterranea* to several different carcinogenic compounds did not lead to tumorigenesis, it has been postulated that the neoblast population and thus the regeneration capacity plays a role in the circumvention of tumor formation. In this study we intend to identify genes that are important in the suppression of tumor formation in planarians, as well as its mechanisms of action.

First of all, in previous research, a non-hypothesis driven open screening study was performed with Cd in order to identify proteins that play a role in the circumvention of carcinogenesis. The goal of the study was to further investigate the function of these identified proteins. Considering that the regeneration process is related to carcinogenesis, and because numerous tumor suppressor genes strictly control regeneration, we hypothesized that a knockdown of proposed tumor suppressor genes that were identified as a result of the open screening study could potentially provide more insight in the mechanism of the circumvention of carcinogenesis. In this research project, two of these proposed tumor-suppressing proteins were studied more profoundly, namely GLIPR1 and MMP19. The planarian homologue for MMP19 is matrix metalloproteinase B (Mmpb) [14].

In order to investigate the function of the identified proteins in the circumvention of carcinogenesis, double-stranded RNA (dsRNA) of either *glipr1* or *mmpb* was introduced in planarians by means of microinjections in the gut, leading to the breakdown of the target messenger RNA (mRNA) and thus a knockdown of the respective protein activity. The animals were cut in three parts to induce regeneration, after which the animals were exposed to the carcinogenic compounds $CdCl_2$ or MMS. The main focus was to observe the planarians in order to detect aberrant phenotypes that occurred as a result of the knockdown of Glipr1 or Mmpb. Since tumor-like outgrowths were detected, the goal was to determine the tumor incidence, as well as to further characterize the outgrowths. Light microscopy was used to evaluate whether or not the phenotypically observed outgrowths consist of tissue, and that they were not merely the result of local fluid buildup, which was also important in order to be able to determine the percentage of tumor occurrence correctly. Furthermore, light microscopy was used to determine the origin of the specific tissue the tumor consists of and to evaluate whether or not the outgrowths showed characteristics of cancer, such as invasions into neighboring tissues. Since little was known about the morphology and anatomy of *S. mediterranea* in light microscopic specimens, the first focus was on studying control animals, after which the outgrowths could be investigated as well. An anti-phospho-histone H3 staining was performed in order to evaluate whether or not the tumor cells were actively dividing, which would be the case in a tumor if it were cancerous.

2. Materials and methods

2.1 Model organism

An asexual strain of *Schmidtea mediterranea*, kindly provided by Stijn Mouton of UMC Groningen, was used in this research project. The animals were maintained in the dark at a constant temperature of 18ºC in deionized doubly distilled water, or in ultra pure water (VWR International, Leuven, Belgium), enriched with 1.6mM NaCl (Merck Millipore, Overijse, Belgium), 0.1mM KCl (VWR International), 1.0mM $CaCl_2$ (UCB Pharma, Anderlecht, Belgium), 0.1mM $MgCl_2$ (UCB Pharma), 1.0mM $MgSO_4$ (VWR International), and 1.2mM $NaHCO_3$ (Acros Organics, Geel, Belgium). The animals were fed with veal liver each week for four hours, after which their medium was changed. The animals were not starved prior to the start of the experiments, except for in the 2D-DiGe analyses, where the animals were starved for one week prior to the start of the experiment.

2.2 Experimental set-up

2.2.1 2D-DiGe analysis

First of all, in order to identify possible tumor suppressor genes, an experiment was performed where intact or regenerating animals were either exposed to 10µM $CdCl_2$ or to 0µM $CdCl_2$. Intact animals were amputated one week before the start of the experiment, while regenerating animals were amputated at the start of the experiment. As a consequence, the intact animals had regenerated their 'lost' body parts prior to the start of the experiment. On the contrary, regenerating animals were forced to regenerate during exposure to 0µM or 10µM $CdCl_2$. The proteins that were altered at the moment of Cd-induced hyperproliferation were detected and quantified by usage of the 2D Quant Kit (GE Healthcare, Diegem, Belgium). As a result of the open screening study with 2D-DiGe, 476 protein spots were identified that were altered as a result of cadmium exposure. Of these 476 protein spots, 251 proteins could be identified. Two proteome databases were consulted in order to match the liquid chromatography–mass spectrometry spectra of these 251 proteins with their respective amino acid sequences. 172 unique proteins were identified, of which ten were considered possible tumor-suppressor proteins. Of these ten possible tumor-suppressor proteins, two proteins were selected for study during this research project, based on their relevance in the control of Cd-induced hyperproliferation, namely GLIPR1 and MMP19. The planarian homologue for MMP19 is matrix metalloproteinase B (Mmpb), as found on NCBI [22]. The *S. mediterranea* homologue for GLIPR1 was found by following certain steps. Sequences of the gene homologues of other model organisms (*Schistosoma mansoni*, *Caenorhabditis elegans*, *Danio rerio*, *Drosophila melanogaster*, *Mus musculus*, *Rattus norvegicus*, *Homo sapiens*) were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and blasted in both Planmine (http://planmine.mpi-cbg.de/planmine/begin.do) and SmedGD

(http://Smedgd.neuro.utah.edu/). The sequences with the lowest E-values were then blasted back to see whether or not it had properties similar to the properties of the gene of interest (GLIPR1). In case a correct sequence was found, potential primers were designed.

2.2.2 Micro-injections

Genes of interest (*glipr1*; *mmpb*) were knocked down in order to test their function in the process of carcinogenesis of regenerating animals, by means of RNAi. Design of the primers was performed by the use of the Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). The following characteristics of the designed primer were important in order to find the best primer pairs: a primer length ranging from 18 to 25 base pairs, an amplicon length of 500 to 800 base pairs, a primer melting temperature of 55-65ºC, a G/C content of closest to 50%, a maximal local alignment score of 8, and a maximal 3'-anchored global alignment score or 3. The importance of the last five bases of each primer was also emphasized, where a high amount of G/C bases was desired. The dsRNA probes were produced by the T7 RibomaxTM Express RNAi system (Promega Corporation, Leiden, Netherlands), according to the manufacturer's instructions. The details for the primers and respective probes are enlisted in table 1. The dsRNA was administered to the animals by means of a Nanoject II injector (Drummond Scientific, Broomall, PA, USA). Each animal was injected three times, 32nl of 1µg/µL dsRNA each, for three consecutive days. Control animals were injected with ultra pure water (VWR international) The injection site was located in the anterior branch of intestines, just anterior to the pharynx. The amputations were performed one day after the third day of injections.

Table 1: RNA sequences of the RNAi probes. The names, abbreviations and accession numbers of the genes of interest are enlisted, along with its respective sense forward (sf), antisense forward (asf), sense reverse (sr), and antisense reverse (asr) primers. The length of the RNAi probes is also given in number of base pairs (bp).

Name	Abbreviation	Accession number	Primer	Primer DNA sequence	Probe length (bp)
glioma pathogenesis-related protein 1	*glipr1*	uc_Smed_v1_Contig176	sf	GGATCCTAATACGACTATAGGGGTCCGAGAAGACTTTCTAACCG	510
			asf	TCCGAGAAGACTTTCTAACCGC	
			sr	GTCCGTCATTGCATGTAGAACC	
			asr	GGATCCTAATACGACTCACTATAGGGGGTCCGTCATTGCATGTAGAACC	
matrix metalloproteinase B	*mmpb*	HE998773.1	sf	GGATCCTAATACGACTCACTATAGGGGCTCATGCGTTTTTCCCTACC	765
			asf	CTCATGCGTTTTTCCCTACC	
			sr	CCAGATAAAAATCCCCATCC	
			asr	GGATCCTAATACGACTCACTATAGGGGCCAGATAAAAATCCCCATCC	

2.3 Phenotypic screening

A $CdCl_2$ stock solution (Acros Organics) of 10,000µM was diluted to a concentration of 10µM with *S. mediterranea* medium. A MMS concentration of 50µM was diluted from a MMS stock solution of 11.8M (Sigma-Aldrich, Diegem, Belgium), by the addition of *S. mediterranea* medium. Exposure to carcinogenic compounds was started 30 minutes post-amputation in head, trunk and tail fragments. The injected animals were maintained in 6-well plates (VWR International), with 3ml of the carcinogenic solutions, or 3ml of *S. mediterranea* medium (0µM $CdCl_2$/MMS). The solutions were refreshed once every week during experiments. By means of a Nikon SMZ 800 trinocular stereomicroscope (Nikon Instruments, Amsterdam, Netherlands), daily observations of the animals were performed for 30 days. Upon observation of atypical phenotypes, these phenotypes were described in detail and animals could be taken for further investigation for either an immunohistochemical staining or a hematoxylin and eosin (H&E) staining. Digital images and movie fragments were acquired using a 1,2" Sony CCD camera (DFK 41AF02, Imaging Source, Bremen, Germany), adhered to the aforementioned stereomicroscope.

2.4 Immunohistochemistry

2.4.1 Mitotic analysis

Since the stem cells of planarians are its only dividing cells, it is possible to evaluate the mitotic activity of stem cells by an immunohistochemical staining using anti-phospho-histone H3 antibodies (Merck Millipore). The anti-phospho-histone H3 antibodies stain phosphorylated serine 10, which is associated with chromosome condensation during mitosis as well as with the G2-phase to M-phase transition in the cell cycle. Digital images (with underlying scale paper) were first taken of animals with atypical phenotypes, as well as of control animals, in order to normalize the amount of stained cells to the body surface of each corresponding animal. Subsequently, these animals were put on ice to stretch the animals. The mucus layer of the animals was removed by immersing the animals in a solution of 5/8 Holfreter (100% Holfreter: 60 mM NaCl (Merck Millipore), 2,4 mM $NaHCO_3$ (Acros Organics), 0,67 mM KCl (VWR International), 1,66 mM $MgSO_4$ (VWR International), 0.9 mM $CaCl_2$ (UCB Pharma)) and 3/8 phosphate buffered saline-triton (PBST) (1 tablet of PBS (Merck Millipore), 200µL Triton X-100 (Sigma-Aldrich), 200ml deionized double distilled water)) with 2% HCl (VWR International) for four minutes. The animals were then fixated by Carnoy's fixative (60% ethanol (Merck Millipore), 30% chloroform (VWR International), 10% acetic acid (VWR International)) for two hours, after which the animals were drenched in 100% methanol (VWR International) and kept at -20ºC until sufficient samples were collected. When sampling was completed, the animals were bleached overnight under a cold lamp in 6% H_2O_2 (VWR International) in methanol at room temperature. The animals were then rehydrated (the methanol was removed) by a series of wash steps in 25%, 50%, 75%, and 100% (twice) of PBST/methanol solutions. Each wash step was performed for ten minutes at room temperature. The animals were subsequently put in 0.1g/ml bovine serum albumin (Sigma-Aldrich), solved in PBST (1%

BSA/PBST) for three hours, by which non-specific binding sites were blocked. The animals were incubated for 44 hours at 4ºC with the primary antibody (anti-phospho-histone H3, biotin conjugate, Merck Millipore), which was diluted 1:600 in 1% BSA/PBST. On day four of the protocol, a series of seven wash steps in PBST was performed in one hour time at room temperature, after which the animals were incubated in 1% PBST-BSA for seven hours. Subsequently, the animals were incubated for sixteen hours at 4ºC in the dark with the secondary antibody (Alexa 568(rhodamine)-conjugated goat anti-rabbit IgG, Merck Milipore), which was diluted 1:500 in 1% BSA/PBS. A series of six wash steps in PBST was then performed in 30 minutes time at room temperature, after which the microscopic slides were prepared by mounting the animals in glycerol, both in the dark. The microscopic slides were examined by means of fluorescence microscopy with a Nikon Eclipse 80i (Nikon Instruments), and pictures were made with a 1.2" Sony CCD camera (DFK 41AF02, Imaging Source). The amount of stained cells was normalized to the body surface, which was determined by measurements of the digital images taken before the start of the protocol, with ImageJ.

2.4.2 Tissue-specific characterization

In order to specify certain tissues seen in the tumor sites, tissue-specific immunohistochemical staining methods can be used. The same protocol is used as was used for the mitotic analysis, except for the usage of different antibodies and an incubation time with the primary antibody of only 24h. For brain tissue, the target of the primary antibody (3C11 anti-SYNORF1, Developmental Studies Hybridoma Bank, Iowa, USA) diluted to a 1:50 concentration in 1% BSA/PBST, is synapsin. The corresponding secondary antibody is Alexa 488(Oregon green)-conjugated goat anti-mouse IgG (Merck Millipore), which is diluted 1:400 in 1% BSA/PBST.

2.4.3 Hematoxylin and eosin staining

Atypical phenotypes with tumor-like outgrowths were evaluated on the presence of living cells by means of a hematoxylin and eosin (H&E) staining and light microscopic observation of the specimens. Special attention was also given to the localization of the tumor as well as their morphological appearance and possible invasions into nearby tissues. Animals with visible anomalous phenotypes, as well as intact and regenerating control animals, were fixated in warm (approximately 45-50ºC) Bouin's fixative (picric acid, acetic acid, formaldehyde) for 24 hours. The next day, the animals were transferred to 70% ethanol, after which they underwent a series of steps with increasing ethanol percentage (75%, 80%, 90% and 100%). The ethanol was removed and replaced by Histoclear. The Histoclear was then replaced by 50:50 Histoclear/molten paraffin mix at 60ºC, and kept for 30 minutes at a constant temperature of 60ºC. The 50:50 Histoclear/molten paraffin mix is subsequently replaced by a 100% molten paraffin mix and incubated for 1 hour at 60ºC. The scaffold was transferred into plastic embedding molds, after which it could be orientated into the required embedding position, while keeping in mind the desired plane of section (sagittal, frontal, transverse). The animal was then embedded into molten wax, after which the sample was held overnight at room

temperature. The following day, the wax had hardened and the plastic molds were removed, after which the sample was sectioned by means of a Leica SM2000 R sliding microtome (Leica Microsystems, Diegem, Belgium), at 10µm. The sections were then stained with hematoxylin and eosin, according to the manufacturer's instructions. After drying, the specimens were inspected by means of a Leica DM2500 microscope (Leica Microsystems), with a Leica DFC450 C camera (Leica Microsystems) mounted. The cell nuclei are stained blue due to hematoxylin staining basophilic structures, which generally contain nucleic acids, while the cytoplasm is stained pink/red due to eosin staining eosinophilic structures, usually composed of intra- or extracellular protein.

3. Results

The effect of a knockdown of the identified proteins that were altered in the open screening study was investigated. Phenotypic changes occurred as a result of a knockdown of Glipr1 or Mmpb. In order to further characterize the observed phenotypes, a light microscopic analysis of the outgrowths was performed, starting with an investigation of control animals. Next, the outgrowths were studied and compared to the control animals. Lastly, a mitotic analysis was performed by means of anti-phospho-histone H3 staining. In this section, the results of the different experiments are presented.

3.1 Glioma pathogenesis related protein 1 knockdown causes phenotypic changes

As previously explained, the membrane protein Glipr1 induces apoptosis and suppresses cell growth in humans. A knockdown of this protein led to the development of local tumor-like outgrowths (Fig. 2), but also to altered pigmentation in all three body fragments (head, trunk and tail), and photoreceptor-regeneration defects in tail fragments, both in Cd-exposed and non-exposed regenerates. After development of local tumor-like outgrowths, these outgrowths were either discarded or the events were followed by the organism's death. After discarding the local outgrowth, the body fragments regenerated normally, and 7 out of the 12 regenerates survived until the end of the experiment. A diminished photoreceptor regeneration or an enlargement of the photoreceptors in tail fragments led to the death of the organism in all cases. The first observed effect in Cd-exposed animals took place earlier than the effect in non-exposed animals (day 1 vs. day 5, respectively), and the outgrowths in Cd-exposed animals were larger than non-exposed animals, although the frequency of occurrence of phenotypic changes was comparable for both conditions, with slightly more events occurring in Cd-exposed animals, relative to the number of animals taken (ratio of 0.236 for Cd-exposed animals vs. ratio of 0.218 for non-exposed animals; Fig. 2). Phenotypic changes were more common in head fragments (ratio of 0.317; Fig. 2), than in trunk (ratio of 0.176; Fig. 2) and tail (ratio of 0.090; Fig. 2) fragments.

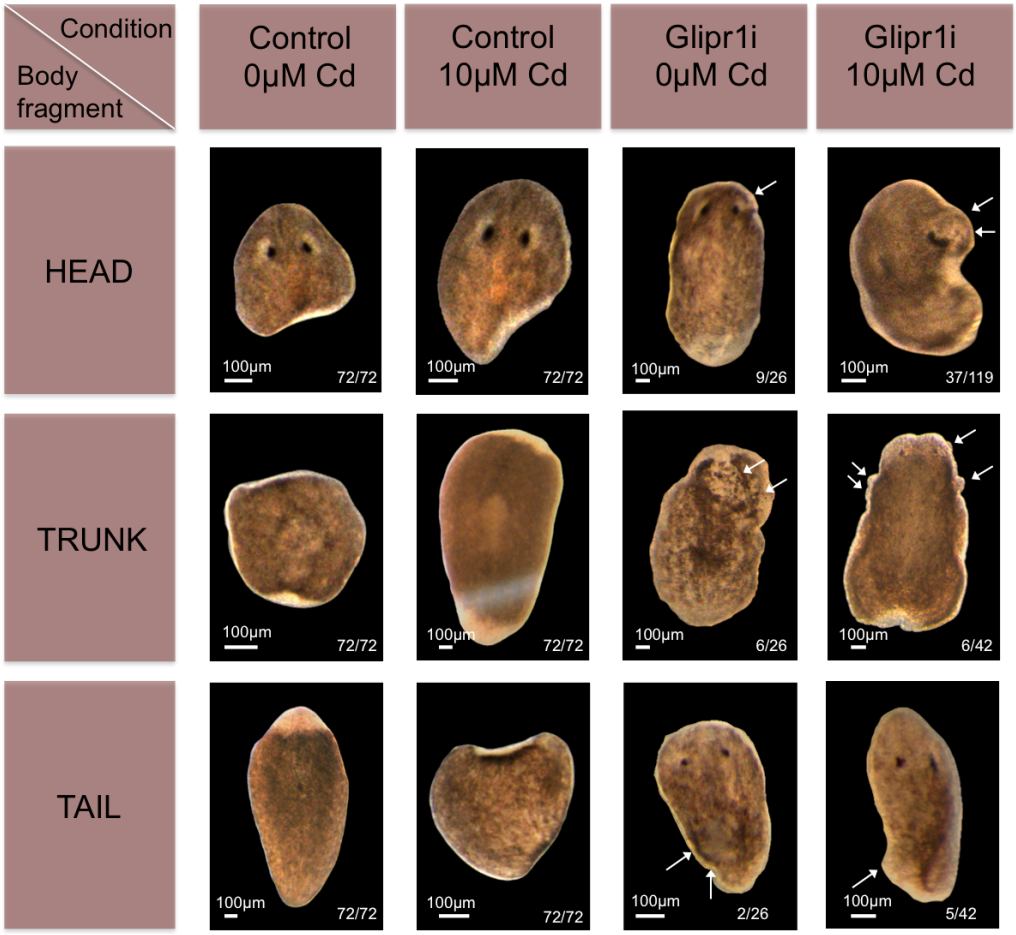

Figure 2: Phenotypic effects observed in head, trunk and tail regenerates of *S. mediterranea* after gene knockdown of Glipr1, in combination with exposure to 0µM $CdCl_2$ or 10µM $CdCl_2$. Orientation of the animals with the anterior end upwards. Ratios of phenotypic changes per condition are shown in the lower right corner. Outgrowths are indicated with arrows. Three times three injections (32nl/injection) were administered on three successive days. The day of the last injection was the first day of exposure to $CdCl_2$. Control animals were injected with ultra pure water. Scale bars represent 100µm.

Table 2: Overview of the different phenotypic effects observed in head, trunk and tail regenerates of *S. mediterranea* after gene knockdown of Glipr1, in combination with exposure to 0µM $CdCl_2$ or 10µM $CdCl_2$.

Body Fragment	Phenotypic observations	Number of occurrence non-exposed	Number of occurrence Cd-exposed
Head	Altered pigmentation	1	8
	Lesions	2	10
	Outgrowths	6	19
Trunk	Altered pigmentation	0	2
	Lesions	2	2
	Outgrowths	4	2
Tail	Outgrowths	1	1
	Photoreceptor regeneration defects	1	4

3.2 Matrix metalloproteinase B knockdown causes phenotypic changes

As previously explained, the protein Mmpb is the *S. mediterranea* homologue of human Mmp19, which is involved in the interaction of cells with their microenvironment as a result of the induction of hydrolysis of the extracellular matrix. A knockdown of this protein led to the development of local tumor-like outgrowths in all three body fragments (head, trunk and tail) (Fig. 3). However, the severity of these outgrowths differed, and three main categories of outgrowths could be distinguished; namely relatively small epidermal protrusions, local relatively small outgrowths, and massive outgrowths. After development of any of the types of tumor-like outgrowths, the organism's death followed. The first observed effect in Cd-exposed animals took place later than in non-exposed animals (day 15 vs. day 9, respectively). Nonetheless, the severity of the outgrowths in Cd-exposed animals and in non-exposed animals was comparable, as was the frequency of occurrence of phenotypic changes for both conditions, with slightly less events occurring in Cd-exposed animals, relative to the number of animals taken (ratio of 0.263 for Cd-exposed animals vs. ratio of 0.278 for non-exposed animals; Fig. 3). Specific for the knockdown of Mmpb was the development of multiple photoreceptors in tail fragments, with the presence of four photoreceptors occurring twice (Fig. 3), however the loss of photoreceptors occurred as well, even as a result of a clear discarding of only the photoreceptor itself (data not shown). Contrary to the development of tumor-like outgrowths, a change in the number of photoreceptors did not lead to the organism's death. Phenotypic changes were more common in trunk fragments (26/74; Fig. 3) than in head (21/74; Fig. 3), and tail (ratio of 13/74; Fig. 3) fragments.

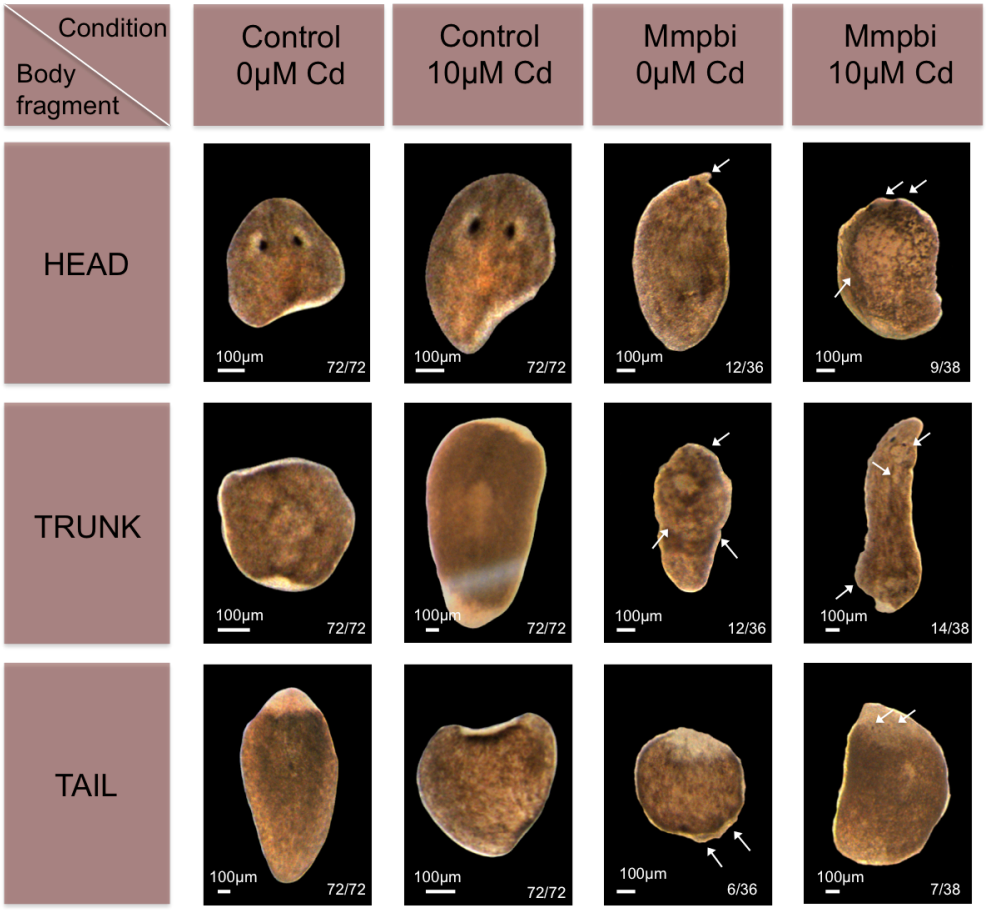

Figure 3: Phenotypic effects observed in head, trunk and tail regenerates of *S. mediterranea* after gene knockdown of Mmpb, in combination with exposure to 0µM $CdCl_2$ or 10µM $CdCl_2$. Orientation of the animals with the anterior end upwards. Ratios of phenotypic changes per condition are shown in the lower right corner. Outgrowths are indicated with arrows. Three times three injections (32nl/injection) were administered on three successive days. The day of the last injection was the first day of exposure to $CdCl_2$. Control animals were injected with ultra pure water. Scale bars represent 100µm.

Table 3: Overview of the different phenotypic effects observed in head, trunk and tail regenerates of *S. mediterranea* after gene knockdown of Mmpb, in combination with exposure to 0µM $CdCl_2$ or 10µM $CdCl_2$.

Body Fragment	Phenotypic observations	Number of occurrence non-exposed	Number of occurrence Cd-exposed
Head	Epidermal outgrowths	4	3
	Massive outgrowths	0	1
	Altered pigmentation	4	4
	Lesions	3	1
	Photoreceptor loss	1	0
Trunk	Local outgrowths	3	2
	Massive outgrowths	6	7
	Lesions	3	5
Tail	Epidermal outgrowths	2	0
	Lesions	0	3
	Photoreceptor gain	2	1
	Photoreceptor loss	2	3

3.3 Light microscopy

The observed tumor-like outgrowths were examined further to be able to conclude that the outgrowths consist of tissue, and not merely for instance of fluid, but also to determine the origin of the specific tissue the tumor consists of and to evaluate whether or not the outgrowths showed characteristics of cancer, such as invasions into neighboring tissues. Hence, the next step in the process was to analyze the tumor-like outgrowths by means of light microscopy. The light microscopic analysis was important to discover more about the function of the knocked-down genes, and more specifically about their role in the circumvention of tumorigenesis in *S. mediterranea*. The animals that showed tumor-like outgrowths were histopathologically examined by preparing specimens via a hematoxylin and eosin (H&E) staining. The localization within the functional tissue, the morphological appearance and the degree of invasion into neighboring tissues of the tumor-like outgrowths was the main focus.

3.3.1 Morphology of control animals

In order to understand more about the morphology of *S. mediterranea* as seen in an H&E staining, control animals were examined first. These animals were either adults or regenerates, which were cut along the sagittal, frontal or transverse plane. Many structures were identified, such as photoreceptors (PR; Fig. 4), tissue of the neuronal line (NT; Fig. 4) with clear ganglions in the anterior end, the basal lamina (ME; Fig. 4) with proximally adjacent the longitudinal and circular muscles, the gastrointestinal tract (GI; Fig. 4) with gastrointestinal cells containing food vacuoles (FV; Fig. 4), the pharynx (PH; Fig. 4), and adhesive glands (AG; Fig. 4) at the posterior end.

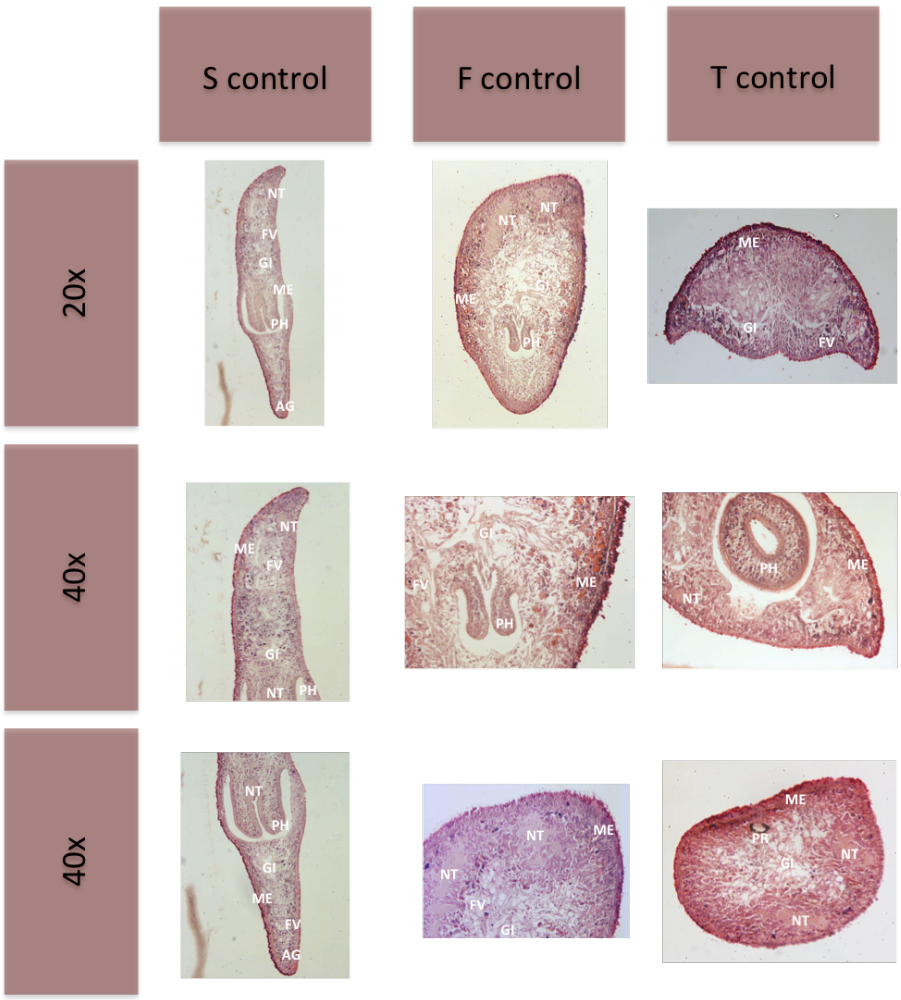

Figure 4: Histological slides of control *S. mediterranea*. Specimens are stained with hematoxylin and eosin. Orientation of the animals with the anterior end upwards for slides cut along sagittal and frontal planes (S control and F control, respectively). Orientation of the animals with the dorsal side upwards for slides cut along the transverse plane (T control). Magnifications of 20x and 40x. Abbreviations: PR: photoreceptors; PH: photoreceptors; NT: nerve tissue; ME: basal lamina; GI; gastrointestinal tract; FV: food vacuoles; AG: adhesive glands.

3.3.2 Aberrant phenotypes caused by glioma pathogenesis related protein 1 knockdown

Tumor-like outgrowths that developed in Cd-exposed *S. mediterranea* regenerates after gene knockdown of Glipr1 were examined by preparing specimens with an H&E staining. The phenotypic picture showed several relatively small "bulbs" across the dorsal side of the animal, anterior to the pharynx (Fig. 5, phenotypic). Corresponding to the phenotypically observed "bulbs", a population of relatively homogenous cells that appeared to be hyperplastic and hypertrophic cells characterized the outgrowths in the light microscopic specimens (Fig. 5, 20x and 100x). After further analysis, by comparing the shape and by examining the location of these cells, it as proposed that the population of cells of which the outgrowths consisted were likely to be gastrointestinal cells.

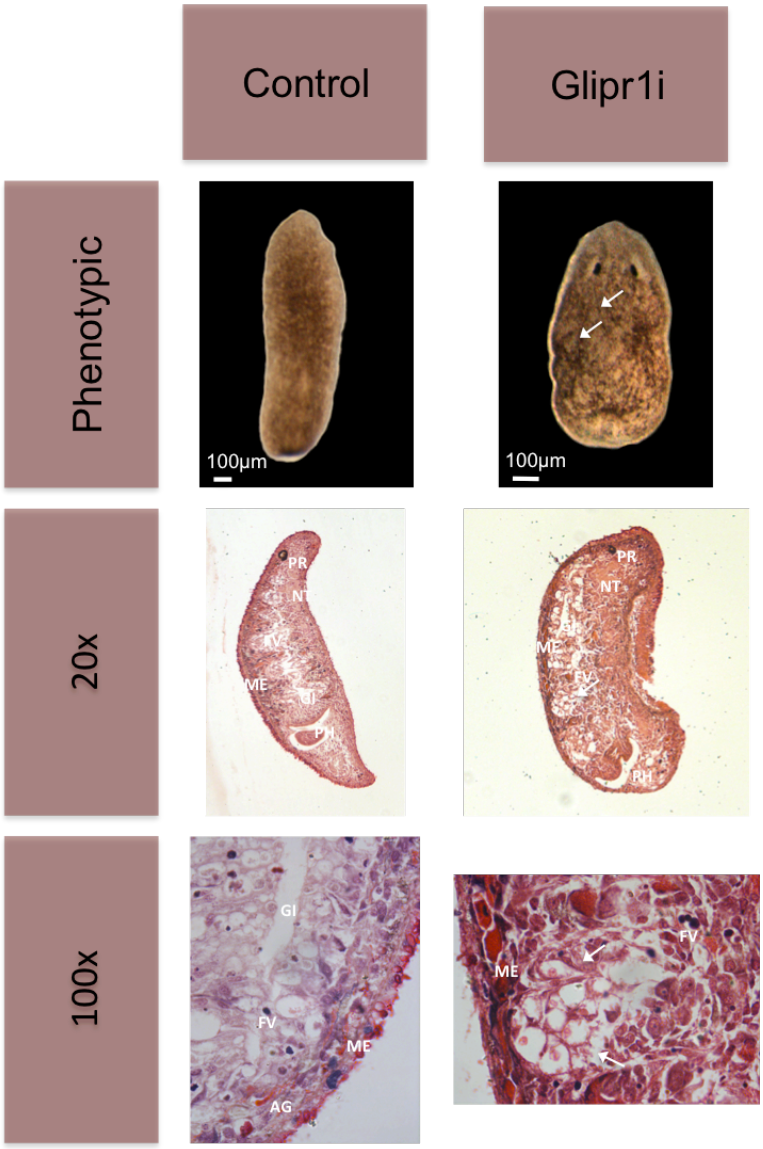

Figure 5: Phenotypic images and histological slides of Cd-exposed Glipr1-knockdown *S. mediterranea*. White arrows indicate outgrowths. Specimens are stained with hematoxylin and eosin and cut along the sagittal plane. Orientation of the animals with the anterior end upwards, dorsal is left. Magnifications of 20x and 100x. Abbreviations: PR: photoreceptors; PH: photoreceptors; NT: nerve tissue; ME: basal lamina; GI; gastrointestinal tract; FV: food vacuoles; AG: adhesive glands. Scale bars represent 100µm.

3.3.3 Aberrant phenotypes caused by matrix metalloproteinase B knockdown

Tumor-like outgrowths that developed in *S. mediterranea* regenerates after gene knockdown of Mmpb were examined by preparing specimens with an H&E staining. Many types of phenotypic changes could be observed after a knockdown of Mmpb, with three categories that could be clearly distinguished, namely local relatively small outgrowths, massive outgrowths and epidermal protrusions.

The phenotypic picture (Fig. 6 (a), phenotypic) showed several relatively small "bulbs" across the dorsal side of the animal, near the anterior end. Corresponding to the phenotypically observed "bulbs", a population of relatively homogenous cells that appeared to be hyperplastic and hypertrophic cells characterized the outgrowths in the light

microscopic specimens (Fig. 6 (a), 20x and 100x). After further analysis, by comparing the shape and by examining the location of these cells, it as proposed that the population of cells of which the outgrowths consisted were likely to be gastrointestinal cells. Furthermore, a disruption of the basal lamina and the epidermis was observed as a result of the presence of the hyperplastic and hypertrophic cells (Fig. 6 (a), 100x).

The phenotypic picture (Fig. 6 (b), phenotypic) showed a large outgrowth coming from the dorsal side of the animal, situated near the pharynx region. This large outgrowth seemed to be populated mostly by cells of the neuronal lineage, forming what looks like a brain ganglion inside the outgrowth as well as a photoreceptor (Fig. 6 (b), 20x and 100x).

The phenotypic picture (Fig. 6 (c), phenotypic) showed small epidermal protrusions at the anterior end of the animal. These epidermal protrusions seemed to be the result of a disruption of the basal lamina, allowing cells to migrate through the disrupted basal lamina and to proliferate between the epidermis and the basal lamina (Fig. 6 (c), 100x).

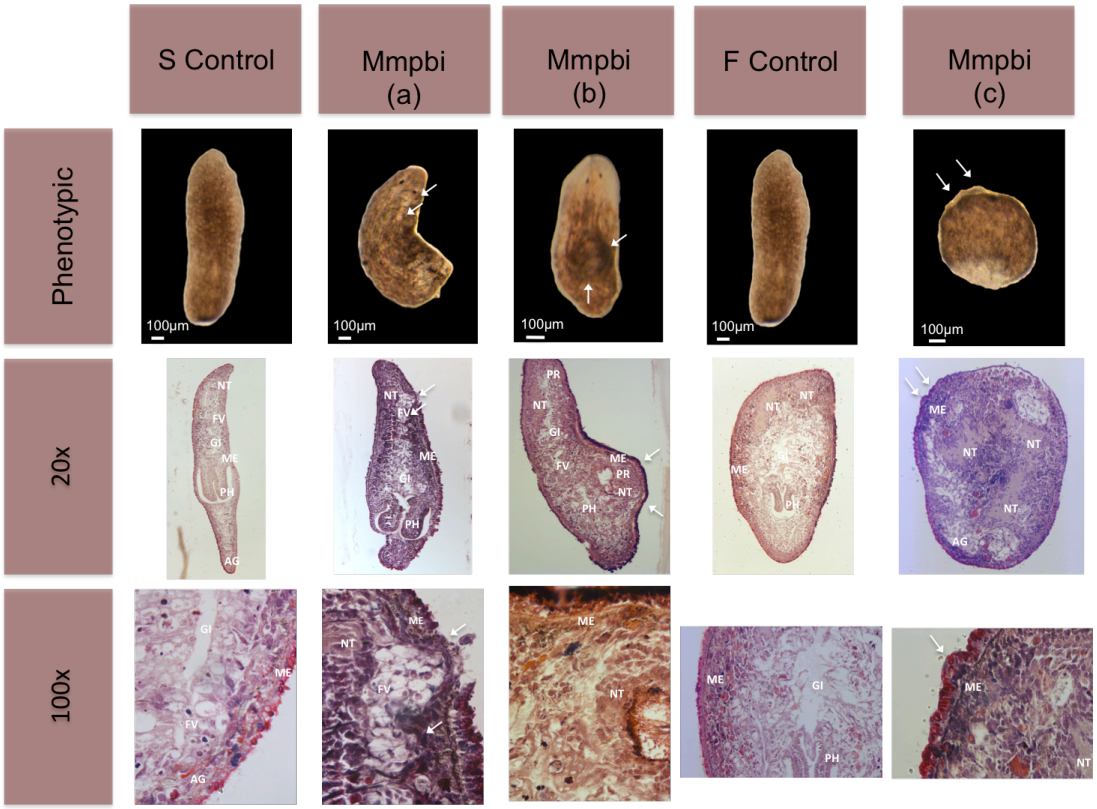

Figure 6: Phenotypic images and histological slides of Cd-exposed Mmpb-knockdown *S. mediterranea*. White arrows indicate outgrowths. Specimens are stained with hematoxylin and eosin and cut along the sagittal plane (first three columns) or frontal plane (last two columns). Orientation of the animals with the anterior end upwards. Magnifications of 20x and 100x. Abbreviations: PR: photoreceptors; PH: photoreceptors; NT: nerve tissue; ME: basal lamina; GI; gastrointestinal tract; FV: food vacuoles; AG: adhesive glands. Scale bars represent 100µm.

3.4 Mitotic analysis

In order to visualize mitotically active neoblasts in the tumor-like outgrowths, an anti-phospo-histone H3 staining was performed on an Mmpb-knockdown animal that was exposed to Cd after being cut and that developed a relatively large dorsal tumor-like outgrowth posterior to the pharynx. As a result, mitotically active neoblasts were found inside an outgrowth, suggesting that it is an actively dividing tissue inside the outgrowth that may cause the outgrowth to occur (Fig. 7). During fixation, the animal was damaged, as seen anterior to the region of the outgrowth, and slightly posterior to the tumor-like outgrowth there was a region that was folded double (Fig. 7, staining).

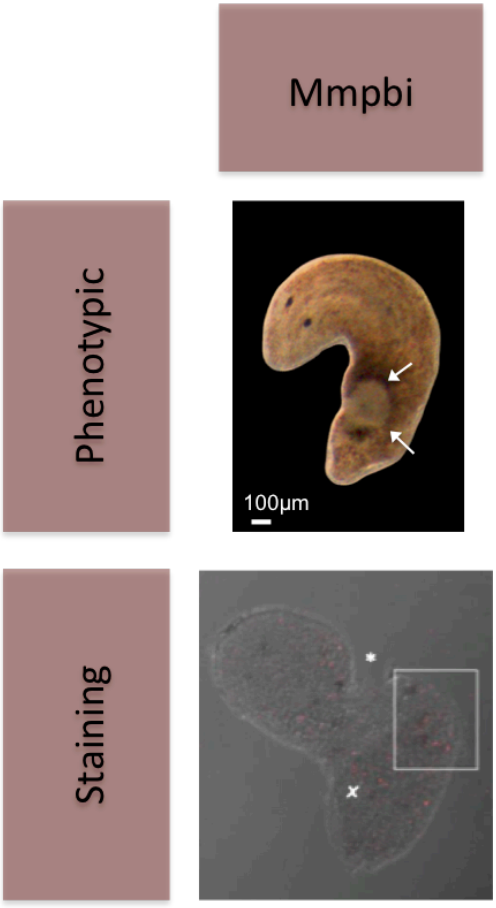

Figure 7: Phenotypic image and immunohistological anti-phospho-histone H3 staining of Cd-exposed Mmpb-knockdown *S. mediterranea*. Orientation of the animals with the anterior end upwards. Phenotypic image: view on the dorsal side, white arrows indicate outgrowth. Scale bar represents 100µm. Image of immunological staining: view on ventral side, white rectangle indicates region of the outgrowth, the animal was damaged during fixation, indicated by an asterisk (*). The animal was folded double in one region, indicated by an x. Combined fluorescent image from confocal stack images with red dots representing mitotically active neoblasts. Magnification of 10x.

4. Discussion

After identifying proteins that showed altered quantities in the open screening study in previous research, the aim of the study was to further examine the function of these identified proteins in the circumvention of carcinogenesis. The most important results, as displayed in the results section, were that a knockdown of Glipr1 and Mmpb leads to the development of tumor-like outgrowths (Fig. 2, Fig. 3). It was found that the tumor-like outgrowths consist of tissue, after which it was even possible to identify and classify these tissues, namely mostly tissues of the gastrointestinal and the neuronal line (Fig. 5, Fig. 6). Another goal of the light microscopic analysis was to detect other signs of carcinogenicity, such as invasions into other tissues. These were not found in this study. In order to conclude that the tumor-like outgrowth consisted of actively dividing cells, a mitotic analysis with an anti-phospho-histone H3 staining was performed, also with a positive outcome (Fig. 7). In this section, the results of the different experiments are discussed in further detail.

4.1 General discussion

Interestingly, it was found that the different body fragments (head, trunk and tail) did not show the same sensitivity towards the development of aberrant phenotypes after gene knockdown. For instance, after a gene knockdown of Glipr1, there were more outgrowths found in head fragments than in any other body fragment of *S. mediterranea* (Fig. 2), which can be the result of the fact that there are no neoblasts anterior to the photoreceptors, diminishing the regeneration potential of the head fragment, thus possibly decreasing the carcinogenesis-circumventing potential [77]. Nonetheless, it is important to note that a delicate balance between the size of the head fragment and the outcome was observed. A head fragment that was cut too small had very little to no regeneration, and about 80% of these regenerates died before twelve days had passed since cutting the animal. A head fragment that was cut too large had less chance of developing a tumor-like outgrowth, probably because it had sufficient neoblasts for the amount of tissue. Hereby, the regenerative capacity was not diminished sufficiently as a result of the absence of neoblasts in the area of tissue anterior to the photoreceptors. To sum up, the neoblasts population in the head part had to be large enough to ensure regeneration, but small enough to increase the chance of developing tumor-like outgrowths due to the fact that there is a region without regenerative potential, enforcing the theory that a vast regenerative capacity is critical in the circumvention of carcinogenesis in *S. mediterranea* and possibly also in other highly regenerative tissues and organisms [53].

Except for differences in the sensitivity of the different body parts on developing tumor-like outgrowths, we have also investigated whether there are specific tissues that are more disposed to advancing into tumor-like outgrowths. It is remarkable that there are two tissues that seem to be more prone to developing tumor-like outgrowths, being tissue of

the gastrointestinal tract and neuronal tissue (Fig. 5, Fig. 6). Although it is known in humans that tissue of the gastrointestinal tract regenerates relatively fast and neuronal tissue hardly regenerates at all, this information is unknown in *S. mediterranea* [78,79]. Because of this, it is not possible to link the occurrence of tumor-like outgrowths in these tissues to their respective duration of the cell cycle, and to compare these to the duration of the cell cycle in other tissues. Of course the dsRNA is taken up through the gastrointestinal tract and it is possible that there is a local buildup of the dsRNA, which could be a reason for the occurrence of hypertrophy and apparent hyperplasia in gastrointestinal cells (Fig. 5, Fig. 6). Taken into consideration that there were more tumor-like outgrowths in the head part, where the two anterior cephalic ganglia are located, the reason why there are tumor-like outgrowths with a mass of neuronal tissue may be the result of the localization of this tissue in the body, and not the result of characteristics of the tissue itself. However, there is neuronal tissue, even including a photoreceptor, found in the large dorsal outgrowth (Fig. 6 (b)), an outgrowth that is not located at the anterior end. Because of the presence of many identified organ-like structures, together with regions within the outgrowth without a clear organization, the large dorsal outgrowth rather resembles a teratoma, a phenomenon that usually occurs more in vertebrates, although it has already been described in *S. mediterranea* after a gene knockdown of P53 as well [80].

Interestingly, although the deviating phenotypes that occurred as a result of a knockdown of Glipr1 of Mmpb were similar in appearance and severity, there was a difference in time of onset (Fig. 2, Fig. 3). Glipr1-knockdown-induced tumor-like outgrowths were observed earlier than Mmpb-knockdown-induced tumor-like outgrowths, probably because of the different functions both genes exert. Glipr1 is a gene that induces apoptosis both dependently and independently from P53, while Mmpb has a role in cell migration and in the composition of the extracellular matrix [21,22,81]. Since the first proliferative peak occurs 6h after cutting *S. mediterranea*, with the major proliferative peak 48-72h after cutting the animal, and because a knockdown of Glipr1 inhibits apoptosis, it is credible that there is a large possibility for a tumor-like outgrowth to arise in this timeframe [82]. Nevertheless, since Mmpb has an effect that involves migration rather than proliferation, it is plausible that there is a delayed onset in tumorigenesis, compared to Glipr1 [22].

It is known that Mmpb exerts an effect on the breakdown of extracellular matrix, which is important for cells to respond to their microenvironment [22]. It is possible that a different microenvironment of stem cells alters the patterning, what has an effect on head-to-tail polarization [83]. The effect on head-to-tail polarization might give rise to the development of head-like structures on regions of the body where these are not supposed to develop, which could be the observed tumor-like outgrowths with neuronal tissue organized as a ganglion and a photoreceptor (Fig. 6 (b)).It is also interesting to note that an Mmpb knockdown caused many other defects concerning the photoreceptors, for instance expelling the photoreceptors or instead producing more photoreceptors, which may also be the result of a defective head-to-tail polarization (Fig. 3). However, Mmpb is also found to be involved in the invasiveness of cancer cells, and an altered

microenvironment can cause a hyperproliferation of neoblasts, with both of these effects being crucial in the development of cancer [22,84]. It is also possible that the photoreceptors were affected by the start of a growing tumor and that this tumor was expelled as such, which can be an explanation for photoreceptor defects as a result of an Mmpb knockdown.

While working on a project, there will always be concerns and items that did not work out as planned. Some remarks on problems I came across during my project will be described in the last paragraph of this section. First of all, planarians have little connective tissues, which is why there are sometimes no clear distinctions between certain structures and tissues [85]. Because of this, and since there are no preceding works performed with light microscopic analyses of *S. mediterranea*, it is important to understand that the appointed tissues in the result section (Fig. 4, Fig. 5, Fig. 6) are not necessarily correct, although these tissues have been compared with light microscopic information drawn from other planarians [86,87]. Secondly, since there were two possible outcomes of the animals after forming a tumor, namely death or expelling the mass, and also because these outcomes occurred in only a few hours time, it is possible that some deviating phenotypes have not been detected. Finally, in regards to the use of light microscopy for the examination of tissues of *S. mediterranea*, it is important to note that there are fixatives that produce better results for ultrastructural analyses. Bouin's fixative is most commonly used in the field as an easy fixative to use in field conditions, although most researchers use glutaraldehyde as a fixative in recent ultrastructural work in the laboratory [88].

4.2 Effect of exposure to different carcinogens

As previously explained, even after a prolonged exposure to genotoxic and non-genotoxic carcinogens, such as MMS and Cd, *S. mediterranea* will not develop tumors, which is fascinating since tumors have been found in other planarians after Cd exposure [61,87,89]. It is already known that Cd exposure leads to hyperproliferation in *S. mediterranea*, however it is suggested that the hyperproliferative response is actually a stem cell response that the planarian uses to circumvent carcinogenesis, although this has not yet been proven and the mechanism of action is unknown [89]. This is in sharp contrast to the effect of Cd in mammals, where Cd exposure leads to an uncontrolled cell growth, which gives rise to the development of tumors [46]. The difference in outcome after Cd exposure could arise from the fact that *S. mediterranea* has a vast regenerative capacity due to the large population of pluripotent stem cells it possesses, which is not seen in mammals [51,69,74]. After identifying proteins that probably play a role in the circumvention of carcinogenesis by performing a non-hypothesis driven open screening study with exposure to Cd in order to unravel the molecular pathway of this process, the idea was to knock down these proteins separately in order to find out what the importance and function of said proteins is, and to subsequently expose the knocked-down animals to different carcinogens, in order to investigate whether the proposed mechanism of circumvention of carcinogenesis would be Cd-specific and whether or not the circumvention of carcinogenesis as a result of exposure to genotoxic carcinogens is

dependent of the same genes as the ones that were identified by the non-hypothesis driven open screening study after exposure to the dubious compound Cd. In these experiments either a Glipr1 or an Mmpb knockdown was performed, which was combined with exposure to either Cd or MMS, or neither. Glipr1 and Mmpb were chosen as target proteins because both proteins were known to play a protective role in the formation of tumors in mammals, were suggested to be involved in the defense against carcinogen-induced carcinogenesis, and were relatively largely affected by the exposure of Cd as seen in the open screening study. Since it is still unclear whether Cd possesses the ability to exert genotoxic or non-genotoxic effects, it is postulated that it induces carcinogenesis both through direct DNA damage (genotoxic effect) as by directly suppressing apoptosis and stimulating cell division (non-genotoxic effect) [44,45]. On the other hand, MMS is a genotoxic carcinogen that works through DNA alkylation, hereby inducing DNA damage, which ultimately leads to carcinogenesis [37].

After a knockdown of Glipr1 or Mmpb outgrowths were observed (Fig. 2, Fig. 3, respectively). Interestingly, these outgrowths occur after gene knockdown of Glipr1 and Mmpb independently of the presence of Cd (Fig. 2, Fig. 3), although they were not observed in MMS-exposed animals (data not shown). Apparently, the importance of Glipr1 and Mmpb in these processes is quite substantial, since a knockdown of these genes causes defects in the regulation of regeneration leading to tumor-like outgrowths in regenerates, without requiring an extra boost on proliferation as a result of the presence of Cd. Another reason why the presence of Cd does not increase the percentage of diverging phenotypes is that, although one gene (Glipr1 or Mmpb) involved in the carcinogenesis-evading response to Cd is knocked down, there are still many other genes that may work independently from the knocked-down gene and that are able to initiate a response in order to circumvent Cd-induced carcinogenesis. On the other hand, the reason why there were no tumor-like outgrowths observed in MMS-exposed animals could be because it is known that an exposure to genotoxic carcinogens initially inhibits proliferation in *S. mediterranea* because of the DNA damage that occurs as a result of the induced DNA alkylation, which is a protective measure of the organism in order to circumvent carcinogenesis [60]. However, after the proliferation stop that is a result of exposure to MMS, the induction of repair mechanisms is initiated. As MMS has different ways in inducing carcinogenesis compared to Cd, the respective repair mechanisms will most likely differ as well [37,44,45,90]. Since direct damage to the DNA can induce carcinogenesis after exposure to MMS, there will be a DNA damage response [37,91]. In order to prevent mutagenesis and possible carcinogenesis as of result thereof, DNA damage will trigger damage sensors that will send a signal through signal transducers, such as the earlier mentioned Nrf2 and Hif-1, to effector cells; a process that will eventually lead to a repair of damaged cells, cell cycle arrest, or, if the DNA damage is too severe, to apoptosis, hereby preventing the division of cells with damaged DNA. The protection of stem cells to DNA damage is critical in highly regenerative organisms in order to preserve the animal from genomic instability [91]. An important repair mechanism pathway for carcinogenesis induced by a genotoxic carcinogen, is the P53-related pathway [92]. Since Glipr1 can work

both dependently and independently from P53, an important tumor suppressor gene, it is possible that the pathway involving the latter gene is important in the repair mechanism for MMS-induced carcinogenesis, although several other pathways are possible as well [21,76,81]. Simultaneously knocking down possible repair mechanisms, for example P53, and Glipr1 in *S. mediterranea* and exposing the animals to MMS could lead to more information about the molecular pathway of the repair mechanism.

Claiming that the observed tumor-like outgrowths that occur as a result of a Glipr1 or Mmpb knockdown could be Cd-specific, since the non-hypothesis driven open screening study was performed with exposure to Cd and thus the proteins that were identified were altered as a response to this carcinogen, seems unlikely because there were also outgrowths observed after the knockdown, in the absence of Cd, as explained earlier. However, it seems that the examined proteins are not critically important in the circumvention of carcinogenesis of chemically induced carcinogenesis as a result of the exposure to MMS, since a knockdown of these proteins and subsequent exposure to MMS did not lead to tumor-like outgrowths.

4.3 Future perspectives

A successful regeneration process is characterized by a well-regulated proliferation, differentiation, migration and apoptosis. When failing to control these four processes, carcinogenesis may occur [1,47]. It is suggested that a high regenerative potential increases the ability to circumvent tumorigenesis, as is seen in *S. mediterranea* [6,7]. Although proven that the outgrowths consist of tissue that actively divides and looks to be showing signs of hypertrophy and hyperplasia (Fig. 5 and 6), it is not yet justified to call this mass of cells cancer. As stated in the introduction, cancer is defined as the uncontrolled division of mutated cells that leads to a malignant tumor [1,2]. Quantifying the division rate of the stem cells inside the outgrowth, compared to the complement regions of the organism can prove interesting to confirm that the outgrowths occur as a result of hyperproliferation. In order to fully characterize the outgrowth as cancer, the outgrowths should be examined further using electron microscopy (EM). By using EM, the cells inside the outgrowths can be evaluated further by examining characteristics of dedifferentiated hyperproliferating cancer cells, such as nuclear hypertrophy, hyperchromatinism, pleomorphism and an increase in number of mitochondria, as well as a greater number of free ribosomes, more in-depth [93]. On the other hand, cancer is malicious and it has been shown that organisms with an outgrowth either expel the outgrowth, or die. Studying the migration of neoblasts towards the outgrowth-site, and investigating the rate of apoptosis inside the outgrowth are next steps necessary to complete the study as all four major parts of both the carcinogenesis and the regeneration processes would be examined.

The regenerative capacity of an organism is clearly important in circumventing carcinogenesis, since it has been shown that regenerating animals show a higher tolerance to carcinogenesis than intact animals [61]. Therefore, the initial main goal was to further investigate the link between carcinogenesis and regeneration by comparing intact adult

animals with actively regenerating animals. After performing a non-hypothesis driven open screening study in which intact animals and regenerates were studied with or without exposure to Cd, several proteins were found that were altered as a result of the presence of Cd. When knocking down two of these genes, Mmpb and Glipr1, *S. mediterranea* was shown to develop tumor-like outgrowths in regenerates. While in the naturally occurring setting intact animals are more prone to develop tumors than regenerates, it may be that regenerates become more prone to developing tumors than intact animals when genes that are important in the regulation of regeneration are knocked down, since the regenerative capacity may be a risk factor in tumor formation when the regulation of regeneration fails [50]. Knocking down Glipr1 and Mmpb in intact *S. mediterranea* would be an interesting next step in this research in order to investigate whether the observed phenotypic disturbances are specific for regenerating animals, or whether these phenotypic disturbances occur in intact animals as well. This information would lead to more knowledge about the link between regeneration and carcinogenesis.

4.4 Conclusion

In this project, two proteins, namely Glipr1 and Mmpb, were studied that are involved in Cd-induced hyperproliferation, which is a regenerative response that was suggested to be a mechanism of circumventing Cd-induced carcinogenesis in *S. mediterranea*. As a result of a separate knockdown of the two genes, tumor-like outgrowths developed in an organism that is otherwise known to be extremely tumor-resistant. It can thus be suggested that these genes exert tumor-suppressing functions, and it is likely that the proteins play a role in the circumvention of carcinogenesis in *S. mediterranea*. Moreover, the cadmium-induced hyperproliferation could indeed be an effective way of circumventing carcinogenesis, which underlies that there is a potential link between regeneration and carcinogenesis. Additionally, it is clear that a knockdown of even one gene can have a detrimental effect in the circumvention of tumorigenesis in *S. mediterranea*, although interplay of many factors such as the effect of the microenvironment can play an important role as well. In order to be sure that the tumor-like outgrowths are cancerous, to conclude that there is a link between regeneration and carcinogenesis, more research has to be performed specifically investigating the characteristics of the cells inside the tumor-like outgrowths by means of electron microscopy.

Discovering exactly how regeneration-related genes aid in circumventing tumor formation and unraveling the cellular and molecular pathways of the process will be important for future treatment options. For instance, stem cells could eventually be engineered in such a way that there is a smaller chance of developing a teratoma, or other cancers, as a result of hyperproliferating stem cells after stem cell transplantation.

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