Biology of Extracellular Matrix 9
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Proteoglycans in Stem Cells

From Development to Cancer

Springer

Biology of Extracellular Matrix

Volume 9

Extracellular matrix (ECM) biology, which includes the functional complexities of ECM molecules, is an important area of cell biology. Individual ECM protein components are unique in terms of their structure, composition and function, and each class of ECM macromolecule is designed to interact with other macromolecules to produce the unique physical and signaling properties that support tissue structure and function. ECM ties everything together into a dynamic biomaterial that provides strength and elasticity, interacts with cell-surface receptors, and controls the availability of growth factors. Topics in this series include cellular differentiation, tissue development and tissue remodeling. Each volume provides an in-depth overview of a particular topic, and offers a reliable source of information for post-graduates and researchers alike.

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ISSN 0887-3224 ISSN 2191-1959 (electronic)
Biology of Extracellular Matrix
ISBN 978-3-030-73452-7 ISBN 978-3-030-73453-4 (eBook)
https://doi.org/10.1007/978-3-030-73453-4

This Springer imprint is published by the registered company Springer Nature Switzerland AG.
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Over the last few decades, stem cell research has taken center stage in the attention of researchers and the public, not least with the awarding of the Nobel Prize for Physiology and Medicine to John B. Gurdon and Shinya Yamanaka in 2012 for their discovery that mature cells can be reprogrammed to become pluripotent (induced pluripotent stem cells, iPSCs). Indeed, stem cells have tremendous potential in research, allowing the generation of various human tissue-specific cell types and lately also organoids. These human research tools were previously impossible to obtain, both in purity and at scale, but based on embryonic stem cells (ES cells) and later iPSC technology, they now serve as useful model systems for basic and disease-oriented research. In the biomedical field, stem cells and aberrant stem cell function have been identified as culprits for disease progression and therapeutic resistance and have therefore become important biomarkers as well as therapeutic targets.

Under physiological conditions, stem cells are characterized by defined properties such as asymmetrical cell division, unlimited proliferative potential, and pluri- or multipotency. These stem cell criteria are in accordance with their role to generate daughter cells which—after a series of differentiation steps—have the ultimate goal of forming a cell type which fulfills a defined function in a multicellular organism, such as a muscle cell, a leukocyte, or a nerve cell. Much has been learned about stem cell function through the study of developmental processes in model organisms. This research has allowed us to identify defined signaling pathways which—via specific interactions of soluble or cell surface-bound ligands with receptors on target cells—convey signals to cells which either promote or inhibit cell differentiation. Indeed, these signals determine if a stem cell remains in a quiescent state, or if it proceeds to differentiate into a specific cell type. Notably, the research efforts of numerous laboratories have revealed that stem cell function is not solely dependent on (stem) cell-autonomous properties and their interaction with growth factors, morphogens, and juxtacrine signaling molecules but also on the extracellular matrix (ECM) within the so-called stem cell niche.

ECM is a complex macromolecular meshwork of structural glycoproteins, proteoglycans, and glycosaminoglycans, adaptor proteins linking these constituents,

and a plethora of functional ligands bound to them. Matrix composition is not only determined by different rates of biosynthesis of individual components but also subject to modification and degradation by specific proteases and glycosaminoglycan-degrading and editing enzymes. Besides providing a structural framework that determines the architectural and mechanical properties of a given tissue, the constituents of the ECM have an essential function in modulating signaling processes with particular relevance to stem cell function. ECM components play pivotal roles by either enhancing or inhibiting the function of ligand–receptor pairs in numerous signaling events, including the WNT, NOTCH, Hedgehog, and FGF-signaling pathways. Moreover, they contribute to proper morphogen gradient formation during development, which adds to the complexity of developmental signaling in cases where an optimal ligand concentration is required to drive a carefully orchestrated morphogenetic process.

We have chosen to focus on proteoglycans and glycosaminoglycans owing to their importance in modulating stemness-associated signaling processes. Glycosaminoglycans are highly negatively charged carbohydrates composed of repetitive disaccharide units which can be attached to core proteins, thus forming proteoglycans. As discussed in this book, heparan sulfate (HS) is a structurally diverse type of sulfated glycosaminoglycan that is ubiquitously expressed on the cell surface and in the extracellular matrix where it interacts with numerous growth factors and morphogens. The association of HS chains with various protein partners is surprisingly unique and dictated by the spatiotemporal expression of various modifying enzymes. Our book highlights the role of specific HS structures generated by different sets of biosynthetic enzymes, and of specific HS proteoglycans of the syndecan and additional proteoglycan families in modulating and orchestrating signaling in the stem cell niche, thus modulating the quantity, quality, and activity of stem cells during development, aging, and disease. Likewise, the role of specific sulfation patterns of the glycosaminoglycan chondroitin sulfate (CS) and of specific CS proteoglycans such as NG2/CSPG4 and versican in development and tissue homeostasis as well as malignant disease will be presented. Furthermore, we will highlight the function of the non-sulfated glycosaminoglycan hyaluronan, and its receptor CD44, a single-chain transmembrane glycoprotein able to modulate several mechanisms that control stem cell behavior, including migration and anchorage, efflux of toxic compounds, growth under hypoxic conditions, and quiescence, besides the properties of self-renewal and differentiation potential. The function of proteoglycans and glycosaminoglycans will be integrated into their interaction with other ECM compounds in this context, including large matrix glycoproteins such as tenascins, enzymes modulating their function such as proteases of the ADAMTS family, and glycosaminoglycan-degrading enzymes such as heparanase and hyaluronidases.

Written by leading experts in the field, the chapters of this book provide a comprehensive overview of the current knowledge on the role of proteoglycans and glycosaminoglycans in stem cell function in physiological processes and disease. Besides describing their role in development, developmental disorders, and physiological functions of embryonic and adult stem cells, several chapters focus on their pivotal role in aberrant stem cell function during neurodegenerative and

malignant disease, thus focusing on major widespread diseases. Indeed, several chapters address the topic of tumor-initiating cells ("cancer stem cells, CSCs"), which are considered a key tumor subpopulation with stem-cell-like properties that may give rise to tumor relapse due to stemness-associated properties such as unlimited proliferative potential, high developmental plasticity, and increased resistance to chemo- and radiotherapy.

The knowledge on ECM function in the stem cell niches and cancer stem cell niches presented in this book comprises a wide range of experimental systems. They range from biochemical and cell biological studies, in vitro studies on embryonic stem cells, induced pluripotent stem cells, and adult stem cells, model organisms including the fruit fly *Drosophila melanogaster*, the zebrafish model, and genetically altered mice as well as pathological studies on human tissues and clinical trials in humans, thus providing a complete view on the topic. Moreover, we will present translational data that mark several ECM constituents of the stem cell niche as an important therapeutic target for malignant, neurodegenerative, and other diseases.

As editors, we hope you will enjoy this new issue of the *Biology of Extracellular Matrix* series, as we believe that the information in this volume will be useful for the research community about the broad range of ECM functions associated with stem cell function in the context of development and disease. We expect that the acquisition of a deeper knowledge in this rapidly evolving field will stimulate new developments in basic, applied, and clinical aspects of stem cell research.

Martin Götte
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Contents

About the Editors

Martin Götte is a Professor at the Department of Gynecology and Obstetrics at the University of Münster, Germany. He obtained his Ph.D. in biochemistry at the University of Göttingen/Max Planck Institute for Biophysical Chemistry in 1997. Following a postdoctoral training at the Department of Cell Biology of Harvard Medical School, and a group leader position at the Institute of Physiological Chemistry of Münster University in 2000, he holds a tenured position as Head of Research in the Department of Gynecology and Obstetrics since 2003. His main areas of research are the role of syndecans, decorin, and glycosaminoglycan biosynthetic enzymes in cancer and inflammation. He is spokesman of the University in the Federal State network for stem cell research and chairman of the board for the reproduction section of the German Society for Endocrinology. He is editorial board member of six Science Citation Index (SCI)-listed journals and has authored more than 160 publications.

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Chapter 1
Heparan Sulfate Proteoglycans in the Stem Cell Niche: Lessons from *Drosophila*

Nanako Bowden and Hiroshi Nakato

Abstract In addition to the obvious importance of stem cells in regenerative medicine, their unique behaviors and ingenious molecular systems for their control are of particular interest in the context of basic cell biology. *Drosophila* genetics plays a pivotal role to reveal fundamental principles of stem cell control mechanisms. Namely, *in vivo* studies using lineage tracing techniques elucidated the cellular and molecular mechanisms of the interactions between stem cells and the extracellular microenvironment. As one of the key components of the stem cell niche, heparan sulfate proteoglycans (HSPGs) have critical functions in regulating stem cell behavior. HSPGs serve as co-receptors for numerous ligands such as fibroblast growth factors, bone morphogenetic proteins, Wnt-related factors, hedgehog, and cytokines, which are all imperative regulators of stem cell behaviors. By modulating and orchestrating these niche factors' signaling and distribution, HSPGs control the quantity, quality, and activity of stem cells.

Abbreviations

BMP	bone morphogenetic protein
CySC	cyst stem cell
Dally	Division abnormally delayed
Dlp	Dally-like protein
Dpp	Decapentaplegic
FSC	follicle stem cell
GSC	germline stem cell
Hh	Hedgehog
Hs6st	HS 6-O sulfotransferases
HSPG	heparan sulfate proteoglycan

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M. Götte, K. Forsberg-Nilsson (eds.), *Proteoglycans in Stem Cells*, Biology of Extracellular Matrix 9, https://doi.org/10.1007/978-3-030-73453-4_1

ISC	intestinal stem cell
NDST	HS *N*-deacetylase/*N*-sulfotransferase
Sfl	sulfateless
Sulf	HS 6-*O* endosulfatase
Trol	Terribly reduced optic lobe
Upd	Unpaired
Wg	Wingless

1.1 Introduction

Stem cells are maintained in specialized microenvironments called "niches," which support their stemness (Spradling et al. 2001). The niche is usually composed of niche cells, extracellular matrix components, and signaling factors emanated from the niche cells. It is poorly understood how each niche factor affects stem cell behavior. For the future development of novel stem cell-based therapies in regenerative medicine as well as drug discoveries for cancers, it is critical to define the molecular mechanisms by which the niche controls stem cell maintenance and differentiation.

The *Drosophila* genetics offers a powerful model system in stem cell biology and has led to the understanding of basic principles of stem cell control. *In vivo* studies using this model have highlighted the importance of various sets of molecules in the stem cell niche. For example, heparan sulfate proteoglycans (HSPGs) were found to be universal niche factors that play critical roles in controlling stem cell behavior (Nakato and Li 2016). HSPGs are a unique class of carbohydrate-modified proteins required for normal cell-to-cell communications. HSPGs serve as co-receptors for a wide array of signaling ligands on the cell surface. Such signaling molecules that require HSPG co-receptors for proper signaling, or HS-dependent factors, include fibroblast growth factors, bone morphogenetic proteins (BMPs), Wnt-related proteins, Hedgehog (Hh), and ligands of the Jak/Stat and EGFR pathways (Nakato and Li 2016). HSPGs generally regulate the activity of signaling receptors in a cell autonomous manner. In some contexts, however, HS expressed by adjacent cells can reach the surface of the neighboring signal receiving cells and affect signaling *in trans* ("*trans*" co-receptor activity) (Jakobsson et al. 2006; Hayashi et al. 2009; Dejima et al. 2011). Additionally, HSPGs control a range and patterns of diffusion of the abovementioned signaling ligands in a tissue (Fujise et al. 2003).

HSPG function is largely controlled by the fine structure of HS chains. HS biosynthesis involves sequential modification events, initiated by *N*-deacetylation and *N*-sulfation of glucosamine residues catalyzed by HS *N*-deacetylase/*N*-sulfotransferase (NDST). This reaction is requisite for subsequent HS modifications, including *C*-5 epimerization of glucuronic acid residues and *O*-sulfation at various ring positions. Following these HS modification steps in the Golgi apparatus, extracellular HS 6-*O* endosulfatases (Sulfs) further modify HS structure on the cell

surface (Dhoot et al. 2001; Morimoto-Tomita et al. 2002; Kleinschmit et al. 2010, 2013). Sulfs specifically cleave 6-*O* sulfate groups on HS. These biosynthetic (by the Golgi enzymes) and post-biosynthetic (by Sulfs) events determine the number of ligand-binding sites on HS as well as the affinity of HS for various ligands. This enables HSPGs to quantitatively regulate signaling output by these factors.

Drosophila has several evolutionarily conserved classes of HSPGs: the syndecan (Sdc), two glypicans called Division abnormally delayed (Dally) and Dally-like protein (Dlp), the perlecan, Terribly reduced optic lobe (Trol), and a new member of the testican family, Carrier of Wingless (Cow). The *Drosophila* model has several unique advantages in proteoglycan biology (Nakato and Li 2016). First, *Drosophila* has all major HSPG core proteins and a complete set of HS biosynthetic/modifying enzymes (Kamimura et al. 2001, 2006), and the complex structural features of mammalian and *Drosophila* HS chains are comparable (Nakato et al. 2019). Second, there is no genetic redundancy in *Drosophila* genes for the HS biosynthetic machinery. This simplifies the interpretation of the genetic analysis of HS functions. Finally, the availability of numerous genetic tools enables us to manipulate HSPG structure and function *in vivo* with a single-cell resolution (Kamimura et al. 2011).

Remarkably, most niche factors thus far identified in different stem cell models are HS-dependent signaling molecules, such as BMPs, Wnts, Hh, and Unpaired (Upd), a ligand of the *Drosophila* Jak/Stat pathway (Hayashi et al. 2012). This fact had suggested that HSPGs may play key roles in stem cell control. Indeed, this was the case. Recent studies established HSPGs as an evolutionarily conserved, universal, and essential niche component, which controls various aspects of stem cell behaviors in many organs through different mechanisms. This chapter focuses on a few genetic studies of *Drosophila* stem cells that approached fundamental problems in stem cell biology: how are the quantity, quality, and activity of stem cells precisely controlled in the niche?

1.2 Heparan Sulfate Proteoglycans Regulate Stem Cell Number in the *Drosophila* Germline Stem Cell Niche

1.2.1 The Drosophila *Female Germline Stem Cell Niche in the Ovary*

The *Drosophila* ovary is the largest organ in the female abdomen with a polarized structure. It contains germline stem cells (GSCs) at the anterior edge, mature oocytes at the posterior end, and progressively developing germline cells in between (Fig. 1.1a). Two or three GSCs are found at the anterior tip of a structure called germarium. The chief component of the GSC niche is a group of somatic cells, the cap cells, which regulate GSC maintenance in a contact-dependent manner (Fig. 1.1b).

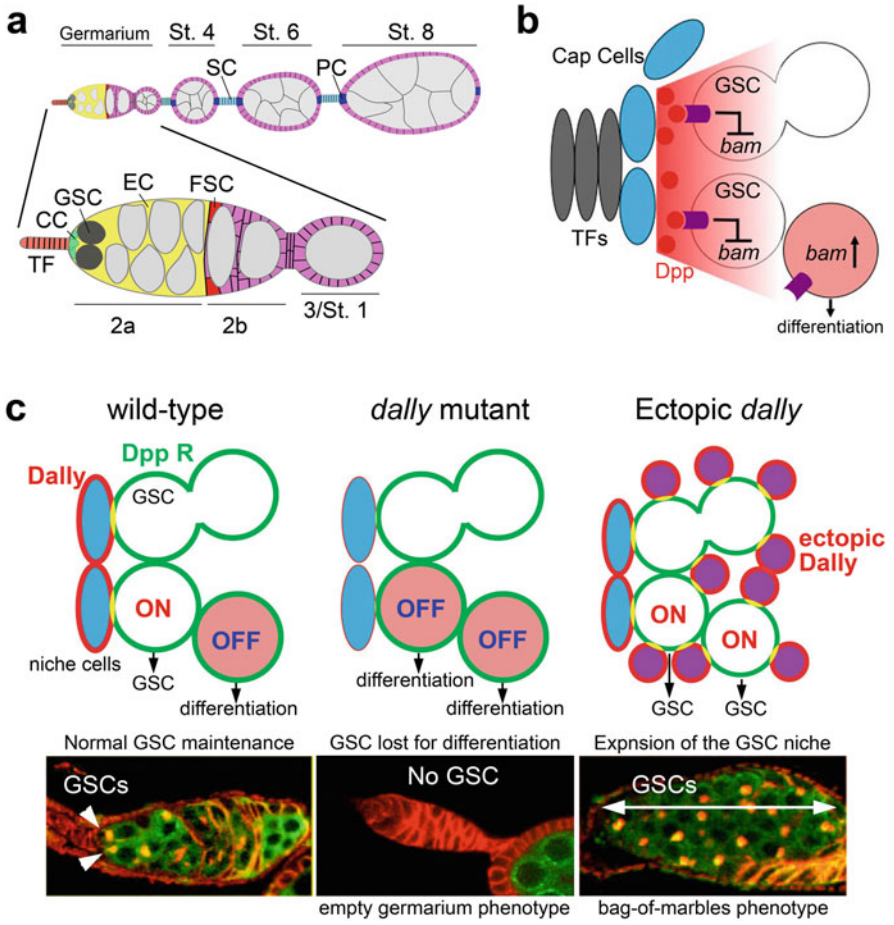

Fig. 1.1 HSPGs in the *Drosophila* female germline stem cell niche. (**a**) The *Drosophila* ovariole. The *Drosophila* ovary is composed of strings of progressively developing egg chambers. Each string is called ovariole (top), which contains a structure called the germarium at its anterior edge (bottom). The germarium contains both GSC (dark gray) and FSCs (red). Different types of somatic cells surround the germline cells: terminal filament cells (TF, orange), cap cells (CC, green), follicle cells (pink), polar cells (PC, blue), and escort cells (EC, yellow). Differentiating germ cells are shown in light gray, and the developmental stages of germ cells are also indicated. Anterior to the left; posterior to the right. (**b**) The GSC niche. When a GSC divides, two daughter cells are produced in a way that one daughter keeps a contact with the cap cells (blue) and the other dissociated from them. The cap cells secrete Dpp (red circle), which represses *bam* only in the contacting GSC daughter. This cell remains a GSC. In the other daughter cell, *bam* directs differentiation into female germ cells. Thus, Dpp regulates GSC fate in a contact-dependent manner. (**c**) Model for the "contact-dependent" regulation of the GSC niche. (left) A dividing GSC (top) produces two daughter cells (bottom). In wild-type, Dally (red) is expressed on the cell surface of the cap cells (blue). Dpp receptors (green) are expressed on germ cells. Dpp activates its receptors at the interface of two contacting cells where receptors and co-receptor "meet" (yellow). Therefore, Dpp signaling is activated in the contacting GSC daughter (ON) but not in the detached daughter (OFF). (middle) In the absence of *dally*, Dpp signaling is impaired. GSC daughters, even when they contact with the cap cells, differentiate, and therefore stem cell populations are lost. This results in the loss of germ cells in a germarium (empty germarium phenotype). (right) Ectopically expressed

The primary molecule that regulates GSC maintenance in the ovary is Decapentaplegic (Dpp), a fly homologue of the BMP2/4 (Fig. 1.1b; Xie and Spradling 1998). Dpp was the first identified niche factor in any stem cell models in all species. Dpp is a highly pleiotropic molecule: it plays critical roles in morphogenesis of many organs throughout development. For example, Dpp acts as a long-range morphogen and regulates patterning along the anterior–posterior axis of the developing wing. In the ovary, Dpp is secreted from the cap cells and activates downstream signaling only in GSCs that directly contact the cap cells. In these cells, Dpp signaling represses expression of *bag-of-marbles* (*bam*). *bam* encodes a translation repressor which acts as a GSC differentiation factor: it is both necessary and sufficient for GSC differentiation (Shen et al. 2009). Since Dpp signaling is activated in a contact-dependent fashion, *bam* is expressed in GSC daughter cells, which have lost contact with the cap cells. These cells differentiate to produce oocytes and nurse cells.

1.2.2 Dally Regulates Stem Cell Number in the GSC Niche

The function of Dpp in the female GSC niche demonstrated that the contact-dependent control system of stem cell fate decision works elegantly in small niches like ones for the *Drosophila* GSCs. However, one crucial question remained unanswered: how can Dpp work in a contact-dependent fashion? Since Dpp is an extensively analyzed, well-known long-range morphogen, it had been a mystery why this molecule cannot act in the non-contacting GSC daughter, which is only a single-cell diameter away from the source of Dpp production.

Dally, a *Drosophila* glypican, was found to be a key player in this regulation (Hayashi et al. 2009; Guo and Wang 2009). In the developing wing imaginal disc, Dally serves as a Dpp co-receptor (Fujise et al. 2003; Akiyama et al. 2008; Belenkaya et al. 2004). In the GSC niche, on the other hand, Dally acts as a "*trans*" co-receptor (Hayashi et al. 2009; Dejima et al. 2011). Dally is specifically expressed on the surface of the cap cells and mediates Dpp signaling *in trans* in a directly contacting GSC (Fig. 1.1c left). In the absence of *dally*, Dpp signaling in these GSCs is compromised. As a result, GSCs are lost to differentiation. As shown in Fig. 1.1c (middle), *dally* mutants show the "empty germarium" phenotype in which germ cells are lost from the germarium. In contrast, when *dally* is overexpressed in somatic cells outside the niche, Dpp signaling is ectopically activated in an expanded area (Fig. 1.1c right). Consequently, GSC-like cells

Fig. 1.1 (continued) dally outside of the niche induces ectopic activation of Dpp signaling throughout the germarium (yellow). Germ cells are maintained as actively dividing "stem cell-like" cells, leading to germline tumors (bag-of-marbles phenotype). The figures are modified from the following publications: Fig. 1.1a (Su et al. 2018) with permission from the Genetics Society of America; Fig. 1.1c (Hayashi et al. 2009) with permission from the Rockefeller University Press

actively divide and expand, resulting in a swollen germarium. This phenocopies the "bag-of-marbles" phenotype caused by loss of the differentiation factor, *bam*.

This ability of Dally to act as a Dpp *trans* co-receptor explains the contact-dependent control of GSC fate decision. Dpp receptors on the surface of GSCs and Dally co-receptor expressed on the cap cell surface can "meet" only at the GSC/cap cell interface, where Dpp activates its receptors. Thus, HSPG *trans* co-receptors act as a key determinant of stem cell number.

1.2.3 The Drosophila *Male Germline Stem Cell Niche in the Testis*

"Asymmetric division," which can produce both differentiating cells and stem cell daughters, is a key feature of stem cells. In some stem cell systems, the asymmetric division is tightly regulated; daughter cells are produced by a precisely oriented division in a way that one daughter cell is kept in the niche while the other daughter is displaced (Yamashita et al. 2010). Such a system is employed in the male GSC niche in the testis (Yamashita and Fuller 2008; Yamashita et al. 2010).

The testis contains two types of stem cells: GSCs and cyst stem cells (CySCs) at its apical tip. CySCs are stem cells for somatic cell populations, and a pair of CySCs enwrap each GSC. A group of somatic cells called the hub function as the niche for both GSCs and CySCs. Like the female GSC niche, the maintenance of stem cells is dependent on the contiguity with the hub. In GSCs, centrosomes are anchored at the hub interface (Fig. 1.2a; Yamashita et al. 2003). A protein complex called the "centrosome anchoring machinery" regulates proper centrosome positioning at the GSC cortex adjacent to the hub (Yamashita et al. 2003; Inaba et al. 2015). In a subsequent step, the daughter centrosome migrates to the opposite side of the cell while the mother centrosome remains in the original position (Fig. 1.2a). This establishes the spindle axis perpendicular to the hub interface and supports the stereotypical asymmetric division of GSCs. Failure in proper anchoring of the mother centrosome near the hub leads to uncontrolled division orientation, which in turn causes aberrant stem cell numbers.

1.2.4 HS in the Niche Regulates GSC Asymmetric Division

Sulfateless (Sfl) is the only NDST gene in *Drosophila*, responsible for the first step of HS modification. Since *N*-sulfation is essential for all subsequent HS modification reactions, loss of *sfl* disrupts biological activities of HS chains (Lin and Perrimon 1999; Lin et al. 1999). Blocking HS biosynthesis by *sfl* RNAi knockdown specifically in the hub of the testes leads to an increase in the number of GSCs. The increased number of GSCs is associated with centrosome mispositioning, leading to

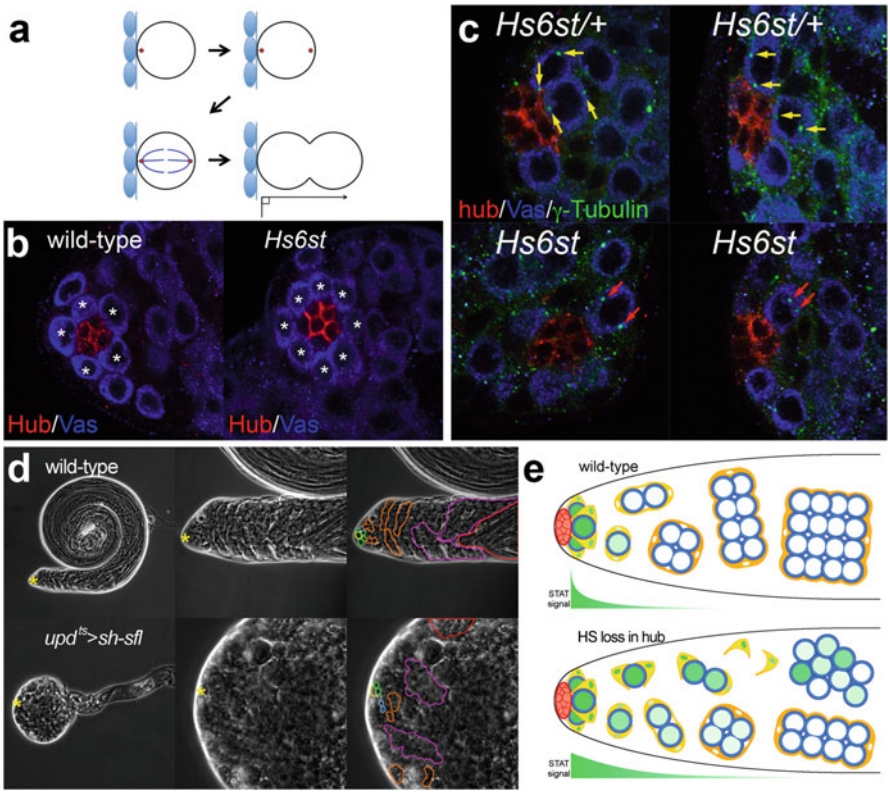

Fig. 1.2 HSPGs in the *Drosophila* male germline stem cell niche. (**a**) In male GSCs, the centrosome anchoring machinery anchors the mother centrosome of a GSC (red dot) near the hub (light blue). The daughter centrosome migrates to the opposite side of the cell, which establishes the orientation of the spindles (blue) in perpendicular to the hub interface. (**b**) HS 6-*O* sulfation regulates GSC number. GSCs (asterisks) are shown for wild-type (left) and *Hs6st* mutant (right) testes. Hub and germ cells are stained with anti-E-cadherin (red) and anti-Vasa (blue) antibodies, respectively. (**c**) *Hs6st* mutation perturbs centrosome positioning in GSCs. Pairs of properly oriented centrosomes (yellow arrows) are shown in *Hs6st/+* testes (top). In *Hs6st* mutants (bottom), the mispositioning of centrosomes (red arrows) is observed. Centrosomes are stained with anti-gamma-tubulin antibody (green). (**d**) Roles of hub HS in the organization of spermatogenic cells. Wild-type (top) and hub-specific *sfl* knockdown (bottom) testes are shown. Two right images are high magnification views of the left image in each row. Different cell types are marked as follows: yellow, hub; green, GSCs; blue, gonialblasts; orange, spermatogonia; pink, primary spermatocytes; red, elongating spermatids. In wild-type, ordered localization of the progressive stages of spermatogenesis is observed. In the absence of hub HS, a severe "tumorous" phenotype with disorganized spermatogenic patterns is observed. Yellow asterisks indicate the hub. (**e**) Model for the role of hub HS in the range of Jak/Stat signaling. CySCs and GSCs are shown in yellow and blue, respectively. (top) In wild-type, HS sequesters Upd ligand at the hub. Sufficient Jak/Stat signaling (green) is activated in stem cells directly contacting the hub. (bottom) In the absence of hub HS, the range with high Jak/Stat signaling is expanded. This produces ectopic populations of stem cells at distant locations from the niche. The figures are modified from the following publications: Fig. 1.2b and c (Levings et al. 2016) with permission from the American Society for Cell Biology; Fig. 1.2d and e (Levings and Nakato 2018) with permission from Oxford University Press

spindle misorientation and symmetric GSC division (Levings et al. 2016). Therefore, besides mediating niche factor signaling, HS expressed in the niche has another role; it regulates GSC number non-cell autonomously by controlling centrosome positioning and spindle orientation.

The 6-*O* sulfate group of glucosamine residues is a key component of the binding sites on HS for many protein ligands both in mammals and in *Drosophila* (Ai et al. 2003; Kamimura et al. 2001, 2006; Kleinschmit et al. 2010, 2013; Wojcinski et al. 2011). In fact, this particular modification event, 6-*O* sulfation, is important for the function of hub HS (Levings et al. 2016). Compared to wild-type testes, the number of GSCs (Fig. 1.2b) and the rate of centrosome mispositioning (Fig. 1.2c) were both significantly higher in *HS 6-O sulfotransferases* (*Hs6st*) null mutants, mimicking *sfl* hub knockdown. Furthermore, overexpression of Sulf1, which removes 6-*O* sulfate groups, specifically in the hub showed the same defects, recapitulating the *sfl* and *Hs6st* knockdown phenotype.

How does hub HS non-autonomously affect the GSC asymmetric division? Analyses of hub-specific *sfl* knockdown and *Hs6st* mutant animals showed that hub HS is required for proper placement and function of the centrosome anchoring machinery (Levings et al. 2016). Therefore, perturbed HS function in the niche leads to a failure in the asymmetric division (centrosome positioning/spindle orientation). This causes a high frequency of symmetric division, which produces two stem cells.

1.2.5 HS in the Niche Prevents Tumor Formation in the Testis

The stem cell niche usually prevents abnormal behaviors of stem cells (Clarke and Fuller 2006; Hudson et al. 2013). Malfunction of niche signaling may predispose a transformation of stem cells into cancerous cells (Bhowmick et al. 2004; Radisky and Bissell 2004), including testicular germ cell tumors (Krausz and Looijenga 2008; Krentz et al. 2009; Gilbert et al. 2011).

A similar phenomenon has been found in *Drosophila* (Levings and Nakato 2018). Hub-specific knockdown of *sfl* causes not only the asymmetric division defect of GSCs described above, but also morphological defects at a lower penetrance. A fraction of hub-specific *sfl* knockdown testes exhibit a severe "tumorous" phenotype (Fig. 1.2d). In the wild-type testis, spermatogenic cells conventionally show the stereotypically ordered, progressive organization. In contrast, this ordered organization is disrupted in hub *sfl* knockdown testes. They often show the "ectopic stem cell" phenotype in which germline and somatic stem cells are abnormally located at distant positions from the hub. These observations indicate that the hub HS has the ability to retain stem cells locally near the niche.

How does hub HS affect the niche–GSC communications? The main pathway responsible for male GSC renewal is Jak/Stat signaling. *In vivo* analyses of Jak/Stat activity showed that hub-specific loss of HS resulted in abnormally higher levels of

Jak/Stat signaling levels in regions distant from the niche (Fig. 1.2e). This observation suggests that hub HS usually sequesters Upd, an HS-binding protein, at the niche. Therefore, a high level of Upd ligand is limited to hub-contacting GSCs. In the absence of hub HS, Upd becomes available in more distant regions, leading to germline tumors. Thus, HS functions as a sentinel to ensure the integrity of the niche organization and prevent tumorigenesis.

1.3 Heparan Sulfate Proteoglycans Regulate Stem Cell Replacement in the *Drosophila* Follicle Stem Cell Niche

1.3.1 The Drosophila *Ovarian Follicle Stem Cell Niche and Stem Cell Quality Control*

Stem cells are maintained throughout adult life, but they are not immortal cells. Individual stem cells have limited lifespans (Margolis and Spradling 1995). Adult stem cells in the niche are regularly replaced so that the niche is always occupied by young, healthy, and functional stem cells (Xie and Spradling 2000; Ryu et al. 2003; Nystul and Spradling 2007). This stem cell replacement can be achieved by competition for niche occupancy between stem cells and their direct progenitors (Nystul and Spradling 2007, 2010; Jin et al. 2008). This behavior of stem cell progenitors contributes to the stem cell quality control (Nystul and Spradling 2007, 2010).

Drosophila ovarian follicle stem cells (FSCs) have been used to study stem cell maintenance and competition (Sahai-Hernandez et al. 2012). All somatic follicle cells are produced by divisions of two FSCs, each of which is located in separate niches in the germarium (Margolis and Spradling 1995) (Fig. 1.1a). These FSCs are maintained through regular replacement: an FSC progenitor routinely migrates across the germarium and reaches a stem cell in the other niche (Nystul and Spradling 2007, 2010). There, these two cells compete for the niche occupancy, but the molecular basis for this competition is unknown. Through this replacement mechanism, the average half-life of wild-type FSCs is approximately 12 days. The phenomenon of stem cell competition was first observed in *Drosophila*, but it is now found in many stem cell types of different species (Li and Clevers 2010).

FSC competitive behavior can be examined in vivo using a lineage tracing technique (Lee and Luo 2001; Takemura and Nakato 2015). This FSC maintenance assay shows that GFP-labeled wild-type FSCs slowly disappear from the germarium due to normal turnover (Fig. 1.3a; Song and Xie 2003; Vied and Kalderon 2009; Vied et al. 2012; Zhang and Kalderon 2000; Kirilly et al. 2005). If the GFP-marked FSC is mutated in a gene essential for normal competition, the mutant progenitors will disappear more quickly than wild-type FSCs. If a mutation results in hyper-competition, on the other hand, the GFP-positive cells tend to occupy both niches. Once both niches are occupied with the hyper-competitive mutant cells, the GFP-positive progenitors expand into the entire epithelial sheet, which is called

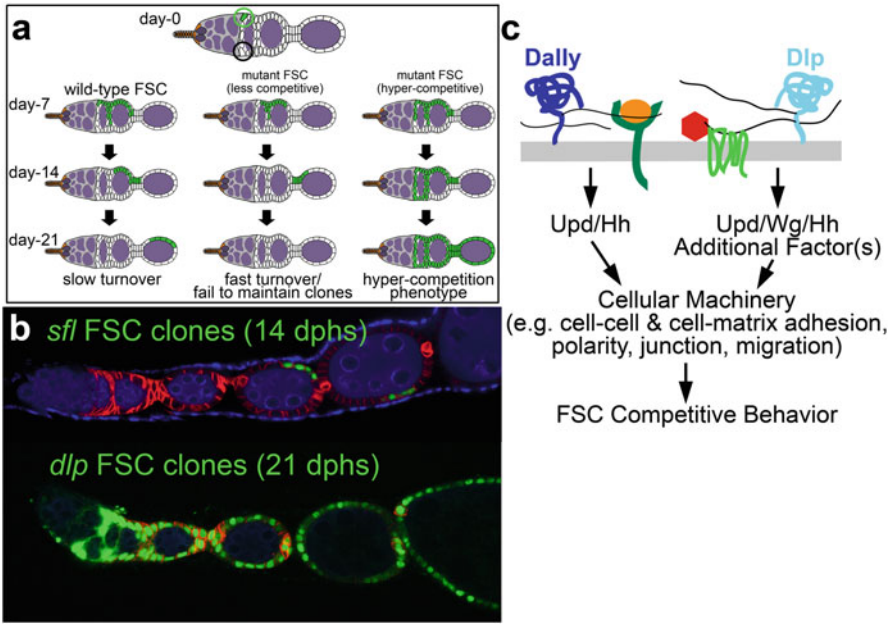

Fig. 1.3 HSPGs in the *Drosophila* follicle stem cell niche. (**a**) FSC lineage analysis. The GFP-positive mutant (or wild-type) FSC and its progenitors are shown in green. Unmarked cells represent wild-type cells. FSC is marked with GFP by heat shock-inducible DNA recombination. Once marked, its progenitors are permanently GFP-positive. Wild-type FSC and its progenitors slowly disappear due to normal turnover (left). If a GFP-labeled FSC is mutant for a gene essential for normal FSC competition, the progenitors will disappear more rapidly than wild-type (middle, less competitive phenotype). If a mutation in an FSC causes hyper-competition, on the contrary, both niches are likely to be occupied by mutant FSCs (right, hyper-competitive phenotype), leading to "all-marked" phenotype. (**b**) Ovarioles with *sfl* and *dlp* mutant FSC clones. (top) *sfl* FSC clones are quickly lost from the germaria. The ovariole bears only few *sfl* mutant progenitors (green) at 14 days after clone induction. *Dally* mutant FSCs show the same phenotype. (bottom) In contrast, *dlp* mutant cells show "all-marked" phenotype. These ovaries do not show any sign of abnormal growth. (**c**) Model for the role of glypicans in FSC competition. Dally and Dlp orchestrate multiple signaling pathways, including Upd, Wg, and Hh. Dlp may regulate additional factor(s). Together, these pathways affect downstream events such as cell adhesion and migration. The figures are modified from Su et al. (2018) with permission from the Genetics Society of America

the "all-marked" phenotype. This behavior of FSC progenitors is similar to the aggressive expansion of cells with pre-cancerous mutations during an early phase of cancer formation (Visvader 2011; Nystul and Spradling 2007; Shiozawa et al. 2011). This assay has been used to identify molecules and pathways that affect FSC replacement and competition (Sahai-Hernandez et al. 2012).

1.3.2 Glypicans Regulate Follicle Stem Cell Competition

In contrast to the non-autonomous functions of HSPGs in the niche cells for GSC control, HSPGs play autonomous roles in the FSCs. Two glypicans, *dally* and *dlp*, are expressed in the FSCs and control FSC competition for niche occupancy (Su et al. 2018). The lineage tracing assay demonstrated that *sfl* or *dally* mutant FSCs disappeared rapidly from the germaria (Fig. 1.3b), showing the necessity of Dally for normal FSC maintenance. Unexpectedly, *dlp* mutant FSCs behaved as a hyper-competitive mutant. *Dlp* mutant progenitors tend to occupy the niche, leading to the "all-marked" phenotype (Fig. 1.3b). Hence, the two *Drosophila* glypicans act in opposite ways during FSC competition and are involved in the FSC quality control. These results are interesting since different human glypican molecules also show opposite effects on cancer formation. Some glypicans are known to be oncogenic, like *dally*, whereas others behave as tumor suppressors, similar to *dlp* (Pilia et al. 1996; Cano-Gauci et al. 1999; Li et al. 2004; Williamson et al. 2007).

What is the molecular basis for the abnormal behaviors of glypican mutant FSCs? At this point, the answer is only partially obtained. FSC maintenance is known to be controlled by Jak/Stat, Hh, and Wingless (Wg: a *Drosophila* Wnt) signaling. The ligands of all these pathways are HS-dependent factors. Systematic analyses of *in vivo* signaling activities showed that *dally* RNAi knockdown in FSCs interfered with Jak/Stat and Hh signaling (Su et al. 2018). Similarly, the Jak/Stat, Hh, and Wg signaling pathways were impaired by *dlp* RNAi knockdown. The reduced Jak/Stat and Hh signaling is consistent with the compromised maintenance and competition of *dally* mutant FSCs (Vied et al. 2012). On the other hand, the mechanism of the hyper-competitive behavior of *dlp* mutant FSC remains to be determined. Interestingly, the overall morphology of *dlp* mutant ovaries is normal. This is a unique phenotype since known mutants that show the all-marked phenotype are typically accompanied by tissue overgrowth. This observation suggests that *dlp* might regulate an HS-dependent pathway that does not promote growth, such as Slit-Robo signaling. As shown in a model figure (Fig. 1.3c), the glypican co-receptors modulate the signaling output of HS-dependent factors, including Upd, Wg, and Hh, and yet unidentified molecules. Combined activities of these signaling pathways appear to consequently control downstream cellular events, such as cell adhesion and migration.

1.4 Heparan Sulfate Proteoglycans Regulate Stem Cell Activity in the *Drosophila* Midgut

1.4.1 The Drosophila *Midgut Intestinal Stem Cells*

Stem cell activity is tightly controlled during regeneration. Upon tissue damage, stem cell mitotic activity is substantially enhanced to replenish lost cells. When the

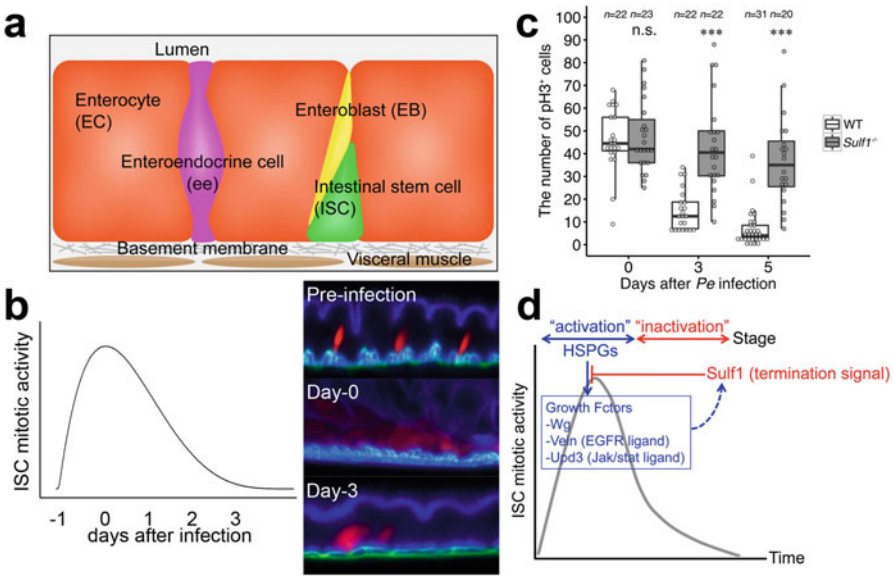

Fig. 1.4 HSPGs in the *Drosophila* midgut intestinal stem cells. (**a**) The *Drosophila* midgut. ISCs are localized at the basal side of the epithelium, contacting the basement membrane. (**b**) Time-course of midgut regeneration after *Pe* infection. (left) Number of mitotically active ISCs at different time points after infection (24 h feeding from −1 to 0). ISC mitotic activity peaks at 0–1 day and drops by day 3. (right) The midgut at pre-infection (top), day 0 (middle; right after the infection), and day 3 post-infection (bottom). *tdTomato* staining shows ISCs and progenitors (red). Cell cortex and the basement membrane are marked in blue (Phalloidin) and green (Trol-GFP), respectively. (**c**) The number of mitotically active cells at indicated time points after *Pe* infection. In *Sulf1* mutants, ISCs remain active for an extended period. (**d**) Model for the midgut regeneration termination. Upon tissue damage, HSPGs promote mitogenic signaling. At this stage, *Sulf1* expression is downregulated. *Sulf1* is re-expressed at the termination stage to reduce the levels of HS 6-*O* sulfation. This results in rapid shutdown of ISC division. Since *Sulf1* is induced by Wg signaling in the developing wing (Kleinschmit et al. 2010), it is possible that *Sulf1* expression at the termination stage is also induced by ISC activating pathways (blue dotted line). Figure 1.4c and d is modified from Takemura and Nakato (2017) with permission from the Company of Biologists

regeneration is completed, their proliferation rate comes back to the baseline. Insufficient stem cell activity results in impaired regeneration. On the other hand, the failure of proper inactivation of stem cell proliferation at the end of regeneration leads to tumor formation.

The *Drosophila* adult midgut has been used extensively to elucidate the molecular mechanisms of epithelial homeostasis and regeneration. The midgut contains two types of differentiated cells: the enterocytes and the secretory enteroendocrine cells (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006). These cells are produced by intestinal stem cells (ISCs), which are found along the entire length of the midgut (Fig. 1.4a; Ohlstein and Spradling 2006; Micchelli and Perrimon 2006). The ISCs increase the rate of division in response to tissue damage (Fig. 1.4b; Amcheslavsky et al. 2009; Jiang et al. 2009). This regeneration can be readily

induced in the laboratory using a variety of chemical and bacterial insults. For example, enteric bacterial infection (*Pseudomonas entomophila, Pe*) activates several pathways, including Jak/Stat, EGFR, Wnt, and Hedgehog (Hh) signaling, to stimulate the repair (Jiang et al. 2016; Guo et al. 2016; Naszai et al. 2015).

Several lines of evidence indicated that HSPGs play a role in midgut homeostasis. The *Drosophila* perlecan, Trol, is required for the attachment of ISCs to the basement membrane and important for ISC maintenance during normal homeostasis (You et al. 2014). Furthermore, loss of an HS 3-*O* sulfotransferase results in the abnormal activation of ISC division *via* upregulated EGFR signaling (Guo et al. 2014). The role of HSPGs in regulating ISCs during homeostasis raised an interesting question: does HS contribute to controlling stem cell activity during regeneration?

1.4.2 HSPGs Regulate Damage-Induced Activation of ISCs

A study using the *Pe*-infection model demonstrated that *sfl* RNAi knockdown in the ISCs blocked the infection-induced increase of ISC division (Takemura and Nakato 2017). As a result, the RNAi animals failed to maintain the normal size and morphology of the midgut, leading to lethality. These observations demonstrated the critical requirement of HS function in ISC activation after bacterial infection. Additionally, silencing *Hs6st* by RNAi knockdown resulted in the same phenotype, showing that HS 6-*O* sulfation is essential for this process. These results are not surprising given that the ligands of mitogenic pathways mentioned above, which promote ISC division in response to tissue damage, are known HS-dependent factors. What is more interesting is a finding that a specific HS modification plays a key role in terminating ISC proliferative activity at the end of regeneration described below (Takemura and Nakato 2017).

1.4.3 Sulf1 Is Required for ISC Inactivation at Late Stages of Regeneration

Not only the activation of the proliferative capacity of stem cells but also its inactivation is equally crucial. The failure of proper termination of tissue regeneration results in the emergence of unwanted cells, leading to aberrant organ sizes (Miyaoka and Miyajima 2013) and an increased risk of cancer (Fuchs et al. 2013; Hsu and Fuchs 2012). Indeed, stem cell activity is precisely controlled at late phases of midgut regeneration. ISC proliferation rates are high for 2 days after *Pe* infection (activation stage) (Fig. 1.4b; Jiang et al. 2009). At this phase, newly emerged cells are stratified and the basement membrane becomes disorganized. The mitotic

activity rapidly decreases on day 3, when regeneration is completed (inactivation stage). By this time, the midgut reforms morphologically normal epithelium.

Quantification of ISC division throughout the course of regeneration revealed that *Sulf1* mutants fail to properly shut down ISC mitotic activity at the termination stage (Fig. 1.4c; Takemura and Nakato 2017). Thus, HS 6-*O* desulfation by *Sulf1* is required for the prompt termination of ISC proliferative capacity at the end of regeneration (Fig. 1.4d). *Sulf1* is secreted from enterocytes and visceral muscles surrounding the midgut epithelium. Interestingly, the expression level of *Sulf1* changes during regeneration; its expression is downregulated soon after regeneration is induced (activation stage) and returns to a normal level at the end of regeneration (inactivation stage). These findings suggested that *Sulf1* acts as a brake for ISC mitotic activity during normal homeostasis and at the regeneration termination. By its activity to remove the ligand-binding sites on HS, Sulf1 contributes to rapid downregulation of mitogen signaling, ensuring appropriate termination.

Despite the significance of the inactivation of stem cell proliferative capacity at the end of regeneration, the molecular basis for this process is poorly understood. One apparent termination mechanism of ISC division is the transcriptional inactivation of the mitogenic signals (Upd3, Vein, and Wg). Sulf1's ability to rapidly shut down multiple ISC mitogen pathways provides an additional control system to halt stem cell division. This finding indicates that HS 6-*O* desulfation plays important roles not only in stem cell control but also in cancer formation. The function of *Drosophila Sulf1* in the midgut is consistent with the involvement of mammalian Sulfs in ovarian, breast, lung, pancreatic, and hepatocellular cancers (Khurana et al. 2013; Lemjabbar-Alaoui et al. 2010; Nawroth et al. 2007; Lai et al. 2003, 2008).

1.5 Concluding Remarks

When two cells communicate, it is obvious that a signaling ligand and its receptor are both required. However, why do such signaling modules typically possess a third component, "co-receptors"? *In vivo* studies of HSPGs, a major class of co-receptors conserved throughout all animal species, using the *Drosophila* model have provided new insights into this classical question. First, during animal development, the signaling outcome is not just ON or OFF, but individual cells must implement a quantitative and precisely controlled signaling dosage. HSPG co-receptors are required for this adjustment: they act as a "rheostat" for fine control of signaling, converting a signaling pathway from a "switch system" to a "dial system." This is reflected by the fact that virtually all known morphogen molecules, which function in a concentration-dependent manner, are HS-dependent factors. Second, HSPGs contribute to positive and negative feedback loops of these pathways, providing the robustness of morphogen signaling, and thus morphogenesis. Again, the robustness is a unique and important characteristic of morphogen systems, although its molecular mechanisms are poorly understood. Finally, HSPGs regulate the spatial distribution of signaling ligands in a tissue as well as the signal reception on the cell

surface. For example, HSPGs concentrate such ligands in the stem cell niche, a small area of a tissue, to help create a special microenvironment for stem cell maintenance. *Drosophila* genetics has revealed that HS-mediated cell communications are indeed effectively used in morphogen signaling and the stem cell niche. Since fundamental principles of HS structure and function are conserved from fruit flies to human, proteoglycan studies using *Drosophila* genetics will provide novel insights into therapeutics and diagnostics for HS-related diseases, such as cancers.

Acknowledgments We thank D. Levings, M. Takemura, E. Nakato, Y. Nakato, and R. Nakato for helpful discussions and critical reading of the manuscript. This work was partly supported by the National Institutes of Health (R35GM131688) to H.N.

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