Secretome analysis by proximal labeling in Golgi apparatus for identifying fat body derived humoral factors in *Drosophila* disc regeneration

Running title: Golgi labeling identifies humoral proteins for regeneration

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Abstract

Humoral factors act as inter-tissue mediators and regulate various organismal physiologies. Although the importance of humoral factors in tissue repair has been recognized recently, our understanding of how humoral proteins regulate tissue repair remains limited. Glycosylation is an important modification of the conventional secretory pathway, and we have demonstrated that N-glycosylation in the Golgi apparatus of the *Drosophila* fat body (FB), the major secretory tissue equivalent to the mammalian liver and adipose tissue, remotely contributes to epithelial tissue repair. To identify humoral factors that contribute to repair via the Golgi apparatus, we constructed a Golgi-specific protein biotinylation system and performed hemolymph proteomics. By combining genetic analyses, we found that FB-derived innate immune regulators and iron mediators affect tissue repair. Altogether, our Golgi-specific labeling system has the potential to identify Golgi-mediated secreted factors that regulate inter-organ communication.

Introduction

In multicellular organisms, the functions of each organ are highly specialized and coordinated. Various tools for analyzing inter-organ communication have recently emerged. In *in vivo* experimental systems, fluorescent-labeled exosomes are expressed in an organ-specific manner, and inter-organ communication was analyzed using zebrafish embryos(1). A method has also been developed to identify the trafficking of secreted proteins by protein biotin labeling in the endoplasmic reticulum (ER)(2).

Tissue regeneration is a homeostatic mechanism against tissue loss that is observed in specific organs and animals(3, 4). Non-autonomous tissue regulation of repair has also been examined. For example, the vagus-macrophage axis regulates liver regeneration in mice, and platelet-derived serotonin mediates liver regeneration(5, 6). In *vitro* screening showed that myristoylated alanine-rich C kinase substrate (MARCKS)-like protein, a factor secreted from the wound epidermis, is required for the initial cell cycle response during axolotl appendage regeneration(7). Although the mechanisms of regenerative regulation through inter-organ communication have been identified, a comprehensive analysis of secreted proteins contributing to tissue regeneration has not yet progressed at the organismal level. *Drosophila* is one of the model organisms that contributes to the systemic aspects of various phenomena through tissue-specific genetic manipulation. Additionally, imaginal discs in *Drosophila* larvae, the epithelial precursors of adult tissues, have been used as models of tissue regeneration(8). In our previous study, we revealed the contribution of the fat body (FB), a counterpart of the mammalian liver and adipose tissue, to wing disc repair(9–11). We found that kynurenic acid (KynA), a humoral metabolite secreted by the FB, was remotely regulated by tachykinin neurons and was necessary for imaginal disc repair(9, 12). Considering that the FB is a major endocrine organ in flies, we have focused on the FB as a source of humoral proteins regulating disc regeneration.

In this study, we attempted to identify secretory proteins that contribute to tissue regeneration. Most proteins are secreted via the ER-Golgi pathway, referred to as the conventional secretory pathway, and undergo glycosylation in the ER lumen or Golgi apparatus(13, 14). Glycosylation is important for the functioning of secreted factors; however, our understanding of its contribution to tissue regeneration is limited. Therefore, we tested whether glycosyltransferases contribute to wing disc regeneration and found that the inhibition of glycosyltransferases in the FB hampered wing disc repair without any obvious effect on development. Using this finding as a starting point, we conducted a comprehensive search for secreted proteins that contribute to regeneration, using Golgi-derived secretome analyses and RNAi screening.

Results

The N-glycosylation pathway in the ER and Golgi apparatus contributes to disc regeneration.

To induce temporal tissue damage and subsequent regeneration of wing discs, a temperature-sensitive diphtheria toxin A domain (DtA^{ts}) was used for temporal cell ablation and repair in our previous study (10) (Fig. 1A). Briefly, larvae were basically reared at 29 ℃ for stopping cell ablation and temporally reared at 18 ℃ for inducing cell ablation. After hatching, we examined the regenerating ability by observing the adult wing phenotype. To manipulate Gal4/UAS-mediated gene expression in the FB, DtA^{ts} was independently induced by the Q system, another binary system, in the wing pouch (WP) region of the wing disc, which becomes an adult wing through metamorphosis. And we also examined the effects of genetic manipulation on development in the FB by keeping the temperature at 29 ℃. Using this system, we attempted to identify regenerative regulatory proteins secreted from FB.

First, we examined the effects of general secretion from the FB on disc regeneration. A previous study showed that factors involved in the protein transport pathway contribute to the general secretion from the FB(15). Knockdown of several factors involved in the secretory pathway, such as Rab1, Syx18 and Rab11, and endocytosis, such as AP2α and Shibire, caused lethal phenotype, indicating their essential role in the FB during development (Fig. S1A, B, C). In contrast, the inhibition of other factors involved in secretion and endocytosis, such as Sec23, deltaCop, and Tweek, increased the percentage of the worsened wing phenotype (Fig. S1B). Knockdown of Sec23, deltaCOP, and Tweek, which causes regeneration failure, had little effect on normal wing formation (Fig. S1C). These results suggest that protein secretion from the FB to wing discs during ablation contributes to the regeneration of the wing disc, whereas the impairment of some regulators of protein transport causes lethality.

When humoral proteins are secreted via the conventional secretory pathway, most secreted proteins undergo a series of biological steps of glycosylation from the ER to the Golgi apparatus(14). By focusing on the glycosylation process as a characteristic of secretion, rather than on the secretory pathway itself, it was thought that screening with a reduced impact on development would be possible. Therefore, we investigated whether glycosyltransferases contributed to disc regeneration. Because N-glycosylation in *Drosophila* is classified into pauci-mannose and complex types, we examined the contribution of the enzymes before and after branching of the N-glycosylation pathway (Fig. 1B). We observed that FB-specific knockdown of the enzyme, Fused Lobes (Fdl), specifically contributing to pauci-mannose pathway did not affect disc repair. However, FB-specific knockdown of enzymes contributing complex N-glycosylation pathway resulted in a worsened wing phenotype after disc damage (Fig. 1C).

Furthermore, the knockdown of ER glycosyltransferases and the early phase of glycosylation in the Golgi caused regenerative inhibition (Fig. S1D, E). As expected, FB-specific RNAi of glycosyltransferases during normal development had no significant effect on wing formation (Fig. 1D and Fig. S1F). We focused on Mgat2 because it acts on the complex-type N-glycosylation branch and significantly affects the disc-regeneration phenotype (Fig. 1B, C). The inhibition of Mgat2 in the FB with distinct FB Gal4 drives confirmed the knockdown efficiency of *Mgat2* (Fig. S2A, B). Ablation by DtA^{ts} caused a developmental delay, as previously described(10), but RNAi of *Mgat2* did not affect developmental speed (Fig. S2C), indicating that Mgat2 regulates disc repair by affecting repair processes. Gene expression of glycosyltransferases, except for Mgat4b, at 0 hr after injury (After Ablation 0 hr: AA0) and at 6 hr (After Ablation 6 hr: AA6), showed no significant change between ablation and non-ablation conditions (Fig. S3A-E).

Next, we investigated whether Mgat2 in the FB contributed to the remodeling of the wing disc after injury by observing the expression of the morphogen wingless (Wg) and GFP-labeled WP regions. Knocking down *Mgat2* in the FB did not significantly affect the recovery of the area of WP region, but affected Wg re-patterning at 30 hr after injury (After Ablation 30 hr: AA30), a late stage of repair (Fig. 1E, F, S2D). We confirmed that this Wg patterning abnormality was not due to excessive cell death by staining for activated caspase and cleaved Dcp1 (cDcp1) (Fig. S2E, F). In addition, the number of mitotic cells in the early stage of regeneration (AA6) was counted using phospho-histone 3 (pH3) staining, and cell proliferation in the wing disc was reduced upon RNAi of *Mgat2* in the FB (Fig. 1G, H). Consequently, Mgat2 in the FB is required to restore cell proliferation during the early stages of repair.

Constructing a system for biotin labeling in the Golgi apparatus.

To identify regenerative proteins secreted from the FB, we aimed to comprehensively detect secretory proteins using TurboID, a tool that biotinylates proximal proteins within a 10 nm range(16). In a previous study, BirA*G3, an earlier version of TurboID, was expressed in the *Drosophila* ER lumen in a tissue-specific manner, enabling the detection of secretory proteins in the specific tissues of other organs(2). In our study, we focused on the Golgi apparatus because complex N-glycosylation was found to affect disc regeneration (Fig. 1B-D). We analyzed tissue-specific secretory proteins by fusing TurboID with a glycosyltransferase present in the Golgi apparatus. Given that a remarkable regeneration failure phenotype was observed with FB-specific RNAi of *Mgat2* (Fig. 1C), we generated *Mgat2-V5-TurboID* fly by fusing C terminus of Mgat2 with a V5 tag and TurboID (Fig. 2A).

When V5-TurboID was expressed in the salivary gland using *Fkh-Gal4* for observation,

the V5 signal diffused within the cell, whereas the Golgi membrane markers Grasp65-GFP (Cis-Golgi) and Man2-RFP (Medial Golgi) formed puncta (Fig. 2B)(17). In contrast, the expression of Mgat2-V5-TurboID (predicted to localize to the Medial Golgi (18)) resulted in puncta formation, and the puncta of Mgat2-V5-TurboID nearly co-localized in close proximity to Golgi membrane markers (Fig. 2B). Furthermore, we investigated the signal of biotin-labeled proteins and V5 tags in the FB. Unlike lacZ (control), V5-TurboID was expressed in the cytosol and nucleus, whereas biotin-labeled proteins marked with streptavidin were expressed mainly in the cytosol. For Mgat2-V5-TurboID, punctate signals of V5 and biotin-labeled proteins were detected in the cytosol (Fig. 2C). These data indicate that Mgat2-V5-TurboID is present in the Golgi apparatus and can label nearby proteins in the Golgi apparatus.

Analysis of secreted proteins from the FB using Mgat2-TurboID identified a group of proteins responsible for regeneration.

To validate whether Mgat2-TurboID (same as Mgat2-V5-TurboID) can label secretory proteins, we examined *Gnmt-T2A-Gal4>Mgat2-TurboID* expression in the FB and detected biotin-labeled proteins in the hemolymph by western blot analysis. Western blot analysis of biotinylated proteins detected by streptavidin showed that *Drosophila* wandering 3rd instar larvae expressing *Mgat2-TurboID* exhibited labeled proteins in both the FB and hemolymph (Fig.3A, B). Compared to *LacZ* and *TurboID*, labeled proteins in the hemolymph of *Mgat2-TurboID* were much detected, and the banding pattens of *Mgat2-TurboID* closely resembled those of *BirA*G3-ER* (Fig. S4A). Cytosolic α Tubulin were not detected in the hemolymph samples. As for labeled proteins in the FB, *TurboID* strongly labeled cellular proteins. Additionally, *Mgat2-TurboID* and *BirA*G3-ER* showed a similar banding pattern, implying that *Mgat2-TurboID* efficiently labeled the proteins of secretary pathway (Fig. S4A, B).

To identify the proteins specifically secreted from the FB during regeneration, we conducted FB-specific protein labeling and proteomics analysis of the hemolymph and FB at the early stage of disc repair (AA0 and AA6) (Fig. 3D). Western blot analysis indicated no significant difference in labeled proteins detected in the hemolymph or FB at any time point between the ablation and non-ablation groups (Fig. 3A, B). There were also no differences in the amounts of TurboID (TbID) or Mgat2-TruboID (Mgat2-TbID) proteins present in the FB, regardless of the time point or ablation (Fig. 3C). Mgat2-TurboID and BirA*G3-ER showed similarly labeled proteins in FB proteomics, as seen in western blot analysis (Fig. S5A). In the hemolymph samples, Mgat2-TurboID and BirA*G3-ER shared many common factors under all conditions (Fig. S5B). Cellular component and pathway analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov). Cellular component analysis indicated that most of the proteins

labeled by V5-TurboID were present in the cytosol and nucleus under all conditions (Table 1), consistent with the observation that V5-TurboID expression was also observed in the cytosol and nucleus (Fig. 2C). Membrane proteins were the top hits in both Mgat2-TurboID and BirA*G3-ER but not in TurboID in the FB (Table 1). ER-related proteins were also more enriched in both BirA*G3-ER and Mgat2-TurboID, and proteins related to the Golgi apparatus were enriched in Mgat2-TurboID under all conditions (Table 1). These data indicate that secretory pathway proteins were more specifically labeled by Mgat2-TurboID and BirA*G3-ER than by TurboID. In the pathway analysis, N-glycosylation-related terms were more frequently detected in Mgat2-TurboID than in others, whereas terms related to ER function were enriched in both BirA*G3-ER and Mgat2-TurboID (Table 2). In summary, these results suggest that the proteins detected in both Mgat2-TurboID and BirA*G3-ER were relatively similar, whereas Mgat2-TurboID more specifically labeled secretory proteins within the Golgi apparatus.

To identify the proteins secreted from the FB into the hemolymph during regeneration, we compared the labeled proteins, which were two times higher in Mgat2-TurboID than in TurboID (Fig. 3D, Table 3). We identified 20 candidate regenerative secretory proteins from the FB in AA0 and AA6, by focusing on the factors that were undetected in NA but detected only in AA. GO analysis of these regenerative candidate proteins using DAVID revealed the upregulation of factors related to innate immunity and iron homeostasis (Fig. 3E). In addition, we compared the labeled proteins, which were two times higher in BirA*G3-ER than in TurboID (Fig. S5C, Table 3). We identified 14 proteins as regenerative candidate proteins, by focusing on the factors that were undetected in NA but detected only in AA, and GO analysis of these proteins also revealed the upregulation of factors related to iron homeostasis and innate immunity (Fig. S5C, D). These results suggest that the factors contributing to the regeneration of wing discs are related to iron binding and innate immunity.

To confirm the importance of FB-derived candidate regenerative proteins in disc regeneration, we conducted RNAi screening (Fig. 3F, G). Most of selected factors were required for disc regeneration, but especially, FB-specific inhibition of iron or immune related factors, transferrin 1 (tsf1), Fer1HCH, Fer2LCH, PGRP-SD, and necrotic (nec), had negative effects on disc regeneration (Fig. 3F, G). These data indicate a non-autonomous requirement for innate immunity and iron-related factors for disc regeneration.

Discussion

In this study, we found that FB glycosylation was non-autonomously required for disc repair (Fig. 1C). To determine the humoral factors involved in disc repair, we established Mgat2-TurboID, a Golgi-specific labeling tool. This system enabled us to perform secretome analysis

involving the Golgi apparatus and identify secretory proteins crucial for disc regeneration, including innate immune regulators and iron mediators (Fig. 3D-F).

Transferrin (Tsf) is an iron transport protein. Tsf1 can bind ferric iron, and ferritin can store approximately 3,000 ferric ions in the ferritin shell. They transport or uptake iron from various organs(19, 20). The uptake and release of iron by blood cells is involved in tissue injury(21, 22). In injured cell populations, damage mitigation and repair responses via regulation of iron by blood cells proceed locally to the site of injury. In the early stages of injury, macrophages protect themselves from harmful iron released from the injured site and from bacterial growth by absorbing and sequestering iron at the site of injury. Such iron sequestration leads to anemia of inflammation observed in chronic inflammatory diseases such as chronic infections, autoimmune diseases, cancer, and chronic kidney disease(21, 22). Subsequently, during the repair phase, macrophages release iron around the site of repair, stimulating cell proliferation and angiogenesis at the site of injury(22). Thus, in mammalian tissue repair, the process begins with sequestration of iron by macrophages, followed by the subsequent supply of iron. Kupffer cells in the liver, like macrophages, are major producers of transferrin and are central to systemic iron metabolism(22). However, it has not been shown how the regulation of iron metabolism by the liver affects remote tissue repair. In this study, we found that iron metabolism regulators generated through secretary pathway in the FB remotely contribute to tissue repair (Fig. 3). It remains unclear how FB-derived iron regulators affect hemocytes, and future verification is warranted.

The contribution of immunity to tissue damage response and inflammatory regulation in tissue repair has been reported and discussed across species, such as mammals and flies(23, 24). Hemocytes, one of the immune regulatory cells, are recruited to damaged wing discs and subsequently activate the JAK/STAT pathway in the hemocytes and FB(25). However, it remains unclear how the Toll and Imd pathway-associated factors detected in the hemolymph proteomics play their respective roles. Recent *Drosophila* studies have suggested a link between iron and innate immunity. During septic injury, hemolymph iron is sequestered into the FB, a phenomenon known as nutritional immunity(26). Iron uptake during infection in the FB suppresses the growth of pathogenic bacteria. During this defense response, the iron transporter Tsf1 is secreted into the hemolymph to sequester serum iron, depending on the immune signaling of the Toll and Imd pathways. In our study, we found that innate immune factors, such as PGRP-SD, nec, and the iron transporter Tsf1, contributed to disc repair (Fig. 3F). Tsf1 is transcriptionally regulated by Toll and Imd signaling (26), and nec is an inhibitor of Toll pathway(27) and PGRP-SD is an activator of Imd pathways(28, 29). These findings

suggest that iron metabolism may be regulated by innate immune pathway. Further investigation is essential to elucidate the role and relationship between iron mediators and immune signaling in the disc regeneration.

We have reported that kynurenine (Kyn) metabolism in the FB is enhanced in response to tissue injury and that KynA secreted from the FB remotely supports tissue repair (9, 12). Vermilion (mammalian tryptophan 2,3-dioxygenase), the rate-limiting enzyme for Kyn production, is an iron-containing oxygenase(30). Therefore, iron regulation in the FB may affect the activity of heme enzymes such as Vermilion and may contribute to remote tissue repair by regulating iron metabolism in the FB.

Previous studies have examined the role of glycosylation in tissue regeneration, but glycosylation does not always promote regeneration. For instance, inhibition of N-glycosylation hinders axon regeneration in *Caenorhabditis elegans*(31). Contrary, in zebrafish hair cell regeneration, the repair process is enhanced by deficiency of Mgat5, an N-glycosylation enzyme(32). In mouse heart regeneration, a deficiency in N-glycosylation of hFSTL1 promotes regeneration(33). As shown above, glycosylation shows different requirements in tissue repair depending on factors, species, and tissues. Therefore, in this study, we attempted to comprehensively search for the FB-derived secretory proteins that influence disc regeneration using Mgat2-TurboID (Fig. 3D, E). Mgat2-TurboID allowed us to identify humoral factors secreted via the Golgi apparatus. Glycosylation affects not only secretion but also protein structure and activity. In addition to a comprehensive search for secreted proteins, the contribution of glycosylation is necessary to further understand of regenerative factors.

The FB serves as a major endocrine organ, comparable to the liver and adipose tissue in mammals, and is responsible for most protein production through the secretory pathway(34). In this study, we employed a proximity-dependent secretory protein labeling method to investigate the secretary pathway proteins during disc regeneration. In previous studies, labeling enzymes were fused to the ER lumen to create proximity-dependent secretory protein labeling tools such as ER-BioID(35), BirA*G3-ER(2), and ER-TurboID(36–39). Using these tools, most secretory proteins from specific organs have been identified in the hemolymph or blood. We fused the labeling enzyme with a glycosyltransferase in the Golgi apparatus to create Mgat2-TurboID, as our initial focus was on complex-type N-glycosylation in disc regeneration. As a tool for detecting secretory proteins, there was a large overlap in the FB-derived labeled humoral proteins between Mgat2-TurboID and BirA*G3-ER during disc

regeneration (Fig. S5A, B). However, we observed that Mgat2-TurboID labeled Golgi proteins more efficiently than BirA*G3-ER. Furthermore, a higher number of membrane proteins were detected in Mgat2-TurboID than in BirA*G3-ER (Table 1). Considering these observations, our Golgi-specific labeling platform is widely applicable for future studies on inter-organ protein trafficking and membrane protein labeling.

Experimental Procedures

Fly strains and genetics

All flies were reared on saf-instant and oriental corn meal medium (hereafter Saf or oriental food) at 25 °C. The detailed food composition per liter of Saf and Oriental foods is shown in Table 4. The fly lines used in this study are mentioned in Table 5.

Temperature shift protocol for temporal ablation

Embryos were laid at 25 °C for 4 hr, and we defined this time point at after egg laying (AEL) as 0 h. The temperature was maintained at 25 °C and shifted to 29 °C, 24 h after AEL 0 hr. For ablation with $WP^{QF2}>DtA^{ts}$, the temperature was raised to 29 °C for 54 hr, and shifted to 18 °C from the early third instar larval stage. After maintaining an 18 °C for 38 hr, the temperature was returned to 29 °C and the flies were either allowed to pupate and eclose, or were dissected at the time points indicated as 0, 6, or 30 hr After Ablation (AA). For proteomics analyses, developmental speed measurements, and qPCR analysis, larvae without DtA^{ts} were used as the non-damaged control and samples were collected at 0 or 6 h non-ablation (NA), at the same time points as AA.

Wing Size Assessment

The flies were preserved in a mixture of ethanol/glycerin (3:1). We observed wing phenotypes by microscopy and manually classified the wing size into four categories: 1) normal: intact wings; 2) chipped: chipped or crumpled wings having a size >90% of control; 3) mild: wings with phenotypes intermediate between groups Chipped and Severe, 4) severe: wings with a size <30% of normal. When counting the regeneration rate of the wings, we noted the statistical analysis values and number of samples (N) above each genotype bar. To take pictures of wings, we dehydrated flies in 100% ethanol for a few hours to one day, following dissection of wings in 100% ethanol and mounting them in Euparal mounting medium (WALDECK GmbH & Co KG, DIVISION CHROMA, 35358-86). Images were captured with a Leica microscope DM5000.

Immunohistochemistry

Discs, fat bodies, and salivary glands were dissected in PBS and fixed with a 4% paraformaldehyde PBS solution for 20 min. Discs were washed with 0.1% Triton X-100 PBS solution (PBST). Fat bodies and salivary glands were washed with 0.3% Triton X-100 PBST. The antibodies used were as follows: rabbit anti-cleaved Dcp1 (Asp216) 1:200 (Cell Signaling, 9578S), mouse anti-Wg (4D4) 1:500 (Developmental Studies Hybridoma Bank), rat anti-phospho-histone H3 (Phospho S28) 1:500 (Abcam, ab10543), rabbit anti-RFP 1:500 (Abcam, ab62341), and mouse anti-V5 1:500 (Invitrogen, R96025). The secondary antibodies used in this study were as follows: Alexa Fluor antibodies (Thermo Fisher Scientific, A-21202, A-21206, A-21208, A-31571, A-31573, A-48272) and AffiniPure antibodies (Jackson ImmunoResearch, 715-165-150, 711-165-152, 712-166-153) diluted at 1:500. The probes used were as follows: Hoechst 33342 (H3570) and Rhodamine Phalloidin (R415) diluted 1:200 (Life Technologies) and Streptavidin-Cy3 (Jackson ImmunoResearch, 016-160-084) diluted 1:500. Confocal images were captured using a Leica SP8 microscope. Quantification of pH3 positive cells in the WP region was performed using the Fiji software.

Fly RNA extraction and synthesis of cDNA

RNA extraction was performed using the RNeasy Micro Kit (QIAGEN) and the ReliaPrep™ RNA Tissue Miniprep System (Promega) according to the manufacturer's instructions. RT-qPCR was performed according to the protocol for nonfibrous tissues using the ReliaPrep™ RNA Tissue Miniprep System (Promega). Briefly, the fat bodies of three third-instar larvae were dissected in PBS and homogenized in 1.5 mL tubes containing lysis buffer. Then, the samples were stored at -80°C according to the protocol of ReliaPrep™ RNA Tissue Miniprep System (Promega). cDNA was synthesized from 400 ng of total RNA using PrimeScript RT Master Mix (Perfect Real Time) (Takara Bio).

Expression constructs

cDNA samples for construction were prepared according to the RNeasy Micro Kit protocol (QIAGEN) and PrimeScript RT Master Mix (Perfect Real Time) (Takara Bio). In QIAGEN protocol, 6 adults or 6 larvae of *UAS-lacZ*^{RNAi} were homogenized with QIAzol in 150 μL and 350 μL of QIAzol was added and incubated for 30 minutes. Then, the samples were stored at -80°C according to the protocol of RNeasy Micro Kit (QIAGEN).

For constructing *UAS-Mgat2-V5-TurboID*, the isoform RB of *Mgat2* was cloned from the cDNA extracted from adult or third instar larvae of the *UAS-lacZ*^{RNAi} using forward primer 5′ -

AAATCAAAGGATCCCCAAAATGTCGAAAATG

and the reverse primer 5′ -
GGGGATGGGCTTGCCCCTCGTGGCCAGCGTC
UAS-Mgat2-V5-TurboID and *UAS-V5-TurboID* were constructed using the *pAct5-V5-TurboID* plasmid, which was generously provided by Dr. Shinoda of the University of Tokyo (40). The V5-TurboID fragment for UAS-V5-TurboID was amplified from the *pAct5-V5-TurboID* plasmid, by PCR using the forward primer 5′ -
AAATCAAAGGATCCCCAAAATGGGCAAGCCC
and the reverse primer 5′ -
TAGTGGTACCCTCGATCACTGCAGCTTTTC
The V5-TurboID fragment for UAS-Mgat2-V5-TurboID was amplified from the *pAct5-V5-TurboID* plasmid, by PCR using the forward primer 5′ -
GGCAAGCCCATCCCCAACCCC
The reverse primer had the same sequence as that of The V5-TurboID fragment for *UAS-V5-TurboID*.
The amplified fragments were inserted into *pUASz1.0* (DGRC, #1431) vector with *Xho*I restriction using the NEBuilder HiFi DNA Assembly Master Mix. This sequence was confirmed by Eurofins, Inc. The vector was injected into the transgenic *y1 w67c23; P{CaryP}attP40* strain. Transgenic flies were generated by Best Gene Inc.

Quantitative RT-PCR

cDNA was diluted 10-fold with Milli-Q, and RT-qPCR was performed using TB Green™ Premix Ex Taq™ (Tli RNaseH Plus) (Takara Bio) to a total volume of 10 μL. Quantitative PCR was performed using the Quantstudio 6 Flex Real-Time PCR system (Thermo Fisher Scientific) according to the standard protocol provided with the software. The primer sequences are as follows:
RNA pol II Forward primer 5'- CCTTCAGGAGTACGGCTATCATCT,
RNA pol II Reverse primer 5'- CCAGGAAGACCTGAGCATTAATCT ;
Mgat2 Forward primer 5'- ATGTCGAAAATGAGGGGTCGC,
Mgat2 Reverse primer 5'- ACTCCGCATGTAGAAGTTGTTG ;
Fused Lobes Forward primer 5'- TTCATCCTGACGGTGCTCTAC,
Fused Lobes Reverse primer 5'- GTATGGGAAAGCCACTAGCATC ;
Mgat4b Forward primer 5'- CAGTTCGTGATTGGAGTACCC,
Mgat4b Reverse primer 5'- GGATCAGACAGTCCACGGTT ;
Mgat3 Forward primer 5'- ATGCAGATGTGAAAGTGGCTG,
Mgat3 Reverse primer 5'- GTGCTACCGCCTCGTTTACTG.

Western Blotting

Fat bodies and hemolymph from 10-20 larvae were collected. Fat body was collected in 55 μL of RIPA buffer (1 M Tris-HCl, pH 8.0 50 mL / 5 M NaCl 30 mL / 5 g Sodium Deoxycholate / 1 g SDS / 1 L SDS g / NP-40 10 mL / MQ up to 1 L) containing 1x protease inhibitor (cOmplete™, EDTA-free Protease Inhibitor Cocktail [Roche, 16829900]). After centrifugation at 10,000 G, 4°C for 10 min, 45 μL of the intermediate layer were collected and stored at -80°C. Larval hemolymph was collected in 15 μL ice-cold PBS by pinching the epidermis using forceps, then extracts were mixed with 30 μL of PBS with 1x protease inhibitor (cOmplete™, EDTA-free Protease Inhibitor Cocktail [Roche, 16829900]). After centrifugation at 1,000 G, 4°C for 3 min to remove hemocytes, 40 μL of the supernatant were collected and stored at -80°C. After protein quantification using the Pierce™ BCA Protein Assay Kit (Thermo Fisher, 23225), 6 × Laemmli sample buffer (1 M Tris-HCl pH 6.8, 12% SDS, 0.6% bromophenol blue, 15% 2-ME) was added, and samples were boiled at 95°C for 5 min. Samples were separated by 10 % SDS-PAGE. The proteins were then transferred to Immobilon-P PVDF membranes (Millipore, IPVH00010) at 25 V, 2.5 mA for 20 min using the Trans-Blot Turbo Transfer System (Bio-Rad, 1704150). The membranes for streptavidin blotting were blocked with 3% BSA overnight at 4°C. And streptavidin-horseradish peroxidase (HRP) was diluted x 1/20,000 in 3% BSA and treated on the membrane for 1-2 h at room temperature. The membranes for antibody staining were blocked with 4% Difco™ skim milk (Becton, Dickinson and Company, 232100)/TBST for 10 min at room temperature. Primary antibodies were diluted in 4% Difco™ skim milk and treated on the membrane overnight at 4°C. After three washes with TBST at room temperature, the secondary antibody was diluted in 4% skim milk and treated on the membrane for 1-2 h at room temperature. The secondary antibody was removed, washed three times with TBST at room temperature, and then treated with Immobilon Western Chemiluminescent HRP Substrate (Millipore, WBKLS0500). Signals were detected using FUSION SOLO 7S. EDGE (Vilber-Lourmat).

Purification of biotinylated proteins

Biotin food was prepared by adding 1 mM Biotin (FUJIFILM Wako Pure Chemical Corporation, 132-02635) to saf food to a final concentration of 100 μM. Animals were placed in vials containing biotin-containing food for injury experiments. Samples were extracted as described in the western blotting section. Purification of the labeled proteins was conducted as previously described (40) with some modifications. Briefly, 150 μg of biotinylated protein-containing lysate was subjected to FG-NeutrAvidin beads (Tamagawa Seiki Co., Ltd, TAS8848 N1171) purification. FG-NeutrAvidin beads (25 μL, approximately 500 μg) were washed three times with RIPA buffer. Benzonase (Merck, 70746-4)-treated biotinylated

protein samples suspended in 1 mL RIPA buffer were incubated overnight at 4 ℃. Conjugated beads were magnetically isolated and washed with 500 μL of ice-cold RIPA buffer solution, 1 M KCl solution, 0.1 M Na_2CO_3 solution and 4 M urea solution. For LC–MS/MS analysis, the purified samples were washed with 500 μL ultrapure water (#214-01301, Wako) and 500 μL of 50 mM ammonium bicarbonate (AMBC) (ammonium bicarbonate, Sigma-Aldrich, A6141-25G). The samples were then mixed with 50 μL of 0.1% RapiGest (Waters, 186001861) diluted in 50 mM AMBC, and 5 μL of 50 mM TCEP (Sigma, #C4706) was subsequently added. Samples were incubated at 60 ℃ for 5 min and then 2.5 μL of 200 mM MMTS (methyl methanethiosulfonate, Thermo Fisher, #23011) was added. One microgram sequence-grade modified trypsin (#V5111, Promega) was then added for on-bead trypsin digestion at 37 ℃ for 16 h. The beads were then magnetically isolated, and 60 μL of the supernatants were collected. Then, 3 μL of 10% TFA (trifluoroacetic acid, FUJIFILM Wako Pure Chemical Corporation, 206-10731) was added to the supernatants and the samples were incubated at 37 ℃ for 60 min with gentle agitation. The samples were then centrifuged at 20 000 g, 4 ℃ for 10 min. The peptides were desalinated and purified using a GL-tip SDB (#7820-11200, GL Sciences, Shinjuku-ku, Japan) following the manufacturer's instructions. The samples were speed-backed at 45 ℃ for 30 min (Tommy Seiko, CC-105) and dissolved in 25 μL of 0.1% formic acid (Kanto Chemical, 16245-63). The samples were then centrifuged at 20 000 g, 4 ℃ for 10 min, and the supernatants were collected. The peptide concentrations were determined using the BCA assay (Thermo Fisher, 23225). Finally, 250 ng of the purified protein was subjected to LC–MS/MS analysis.

LC-MS/MS analysis

Samples were loaded onto Acclaim PepMap 100 C18 column (75 μm × 2 cm, 3 μm particle size and 100 Å pore size; #164946, Thermo Fisher Scientific) and separated using nano-capillary C18 column, (75 μm × 12.5 cm, 3 μm particle size, #NTCC-360/75-3-125, Nikkyo Technos, Bunkyo-ku, Japan) in an EASY-nLC 1200 system (Thermo Fisher Scientific). The elution conditions are listed in Table 6. The separated peptides were analyzed using QExactive (Thermo Fisher Scientific) in the data-dependent MS/MS mode. The parameters for MS/MS analysis are listed in Table 7. The collected data were analyzed using Proteome Discoverer (PD) 2.2 software with the Sequest HT search engine. The parameters for the PD 2.2 analysis are listed in Tables 8 and 9. Peptides were filtered at a false discovery rate of 0.01 using the Percolator node. Label-free quantification was performed based on the intensities of the precursor ions using a precursor-ion quantifier node. Normalization was performed using the total amount of peptides in all average scaling modes. Proteins with 1.5 or 2 folds higher abundance ratios relative to the lacZ or TurboID controls were considered for further

analysis. The MS proteomics data were deposited in the ProteomeXchange Consortium via the jPOST partner repository with the dataset identifier PXD054313 for FB samples and PXD055017 for hemolymph samples.

Table 6. LC settings. Gradient settings for the LC analysis are presented.

Time	Duration	% acetonitrile	Flow rate ($nL \cdot min^{-1}$)
0	0	0	300
5	5	10	300
60	55	40	300
62	2	95	300
80	18	95	300

Table 7. MS/MS settings. Settings used for the MS/MS analysis are presented.

Method parameter		Value
Polarity		Positive
Full MS	Microscans	1
	Resolution	70 000
	Automatic gain control (AGC) target	3.00 E + 06
	Maximum ion time	60 ms
	Scan range	400-1500 *m/z*
dd-MS2	Microscans	1
	Resolution	17 500
	Automatic gain control (AGC) target	1.00 E + 5
	Maximum ion time	60 ms
	Loop count	10
	Isolation window	3.0 *m/z*
	Fixed first mass	-
	Normalized collision energy	28
dd settings	Intensity threshold	8.30 E + 03
	Charge exclusion	Unassigned, 1, 5-8, >8
	Peptide match	Preferred
	Exclude isotopes	On
	Dynamic exclusion	6.0 s

Table 8. Proteome Discoverer 2.2 settings. Major Proteome Discoverer 2.2 settings for the processing workflow of proteomics analysis are presented.

Name of Node	Name of Parameter	Template	This analysis
Spectrum Files RC	Protein Database	None	Drosophila_melanogaster_uniprot-

			proteome_UP000000803.fasta
	1. Static Modification	Carbamidomethyl / +57.021 Da (C)	Methylthio / +45.988 Da (C)
Sequest HT	Protein Database	None	Drosophila_melanogaster_uniprot-proteome_UP000000803.fasta
	Fragment Mass Tolerance	0.6 Da	0.02 Da
	2. Dynamic Modification	None	GG / +114.043 Da (K)
	1. Static Modification	Carbamidomethyl / +57.021 Da (C)	Methylthio / +45.988 Da (C)

Table 9. Proteome Discoverer 2.2 settings. Major Proteome Discoverer 2.2 settings for the consensus workflow of proteomics analysis are presented.

Name of Node	Name of Parameter	Template	This analysis
MSF Files	Merge Mode	Globally by Search Engine Type	Do Not Merge
Precursor Ions Quantifier	Normalization Mode	Total Peptide Amount	None
	Ratio Calculation	Pairwise Ratio Based	Summed Abundance Based
	Hypothesis Test	ANOVA (Background Based)	ANOVA (Individual Proteins)

Analysis of secreted regenerative proteins based on proteomics data

For the analysis of the Abundance Ratio of the proteomics data, proteins with Mgat2-TurboID/TurboID higher than 2.0 and a P-value less than 0.05 were selected, and extracted candidate proteins detected only injury. The web tool DAVID for annotation, visualization, and integrated discovery (DAVID) (https://david.ncifcrf. gov/) was used.

Statistical analysis

Data are presented as stacked bar graphs, bar charts, or mean ± standard error. To analyze the independence of the cross-tabulation tables used in the stacked bar graphs, p-values were calculated using Fisher's exact test for all data. The unpaired t-test was used to calculate p-values for comparisons between the two groups, and one-way ANOVA, Multiple comparisons

were used to calculate p-values for multiple comparisons of the grouped samples. p-values are NS: not significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$. GraphPad Prism statistical analysis software and RStudio software were used to generate graphs and perform statistical analyses.

Figure Legends

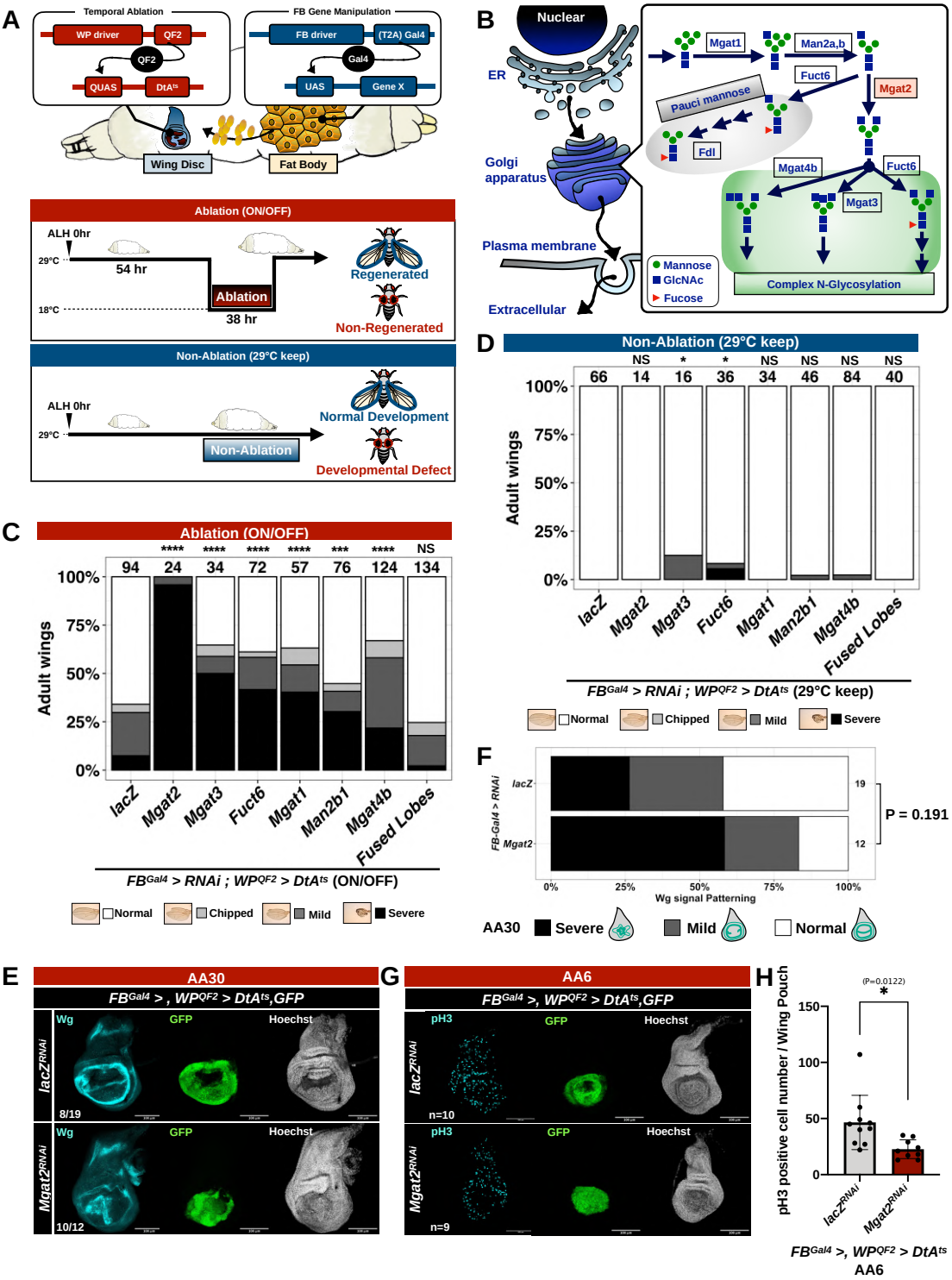

Figure 1 Glycosyltransferase in the FB is necessary for disc regeneration.

(A) Schematic view of temporal disc ablation and gene manipulation in the FB. Temporal disc damage is induced by DtA^{ts} at a low temperature (18 ℃) for 38 hr and returned to 29 ℃ after ablation. This condition is indicated as "Ablation". In non-damaged condition, temperature shift is not induced and kept at 29 ℃ to avoid the induction of damage by DtA^{ts}. This condition is indicated as "Non-Ablation."

(B) N-glycosylation pathway in the Golgi apparatus, in particular before and after the branching of a pauchi mannose and complex N-glycosylation pathways.

(C and D) Comparison of the adult wing sizes between ablation (C) and non-ablation (D). Inhibition of N-glycosylation enzymes hampered disc regeneration. RNAi was induced in the FB by FB^{Gal4}. Statistical analysis was conducted using the Fisher's exact test to compare the control ($WP^{QF2}>DtA^{ts}$; $FB^{Gal4}>lacZ^{RNAi}$) with the treated larvae. NS: not significant, **: $p<0.01$, ****: $p<0.0001$.

(E and F) Representative examples of wing discs developed within the indicated time course. Discs were dissected at 30 hr After Ablation (AA30). FB specific inhibition of *Mgat2* affected Wg patterning in disc regeneration. The discs were stained with anti-Wg antibody and Hoechst33342. White scale bar, 100 μm. Abnormality of Wg patterning in the wing pouch was classified into 3 groups and classification was quantified in (F).

(G and H) Representative examples of wing discs developed within the indicated time course. Discs were dissected at 6 hr After Ablation (AA6). FB specific inhibition of *Mgat2* affected cell proliferation in disc regeneration. The discs were stained with anti-pH3 antibody and Hoechst33342. White scale bar, 100 μm. pH3 positive cell numbers in the wing pouch region was quantified in (H). Error bars indicate standard error of the mean. Unpaired t test was applied. *: $p<0.05$.

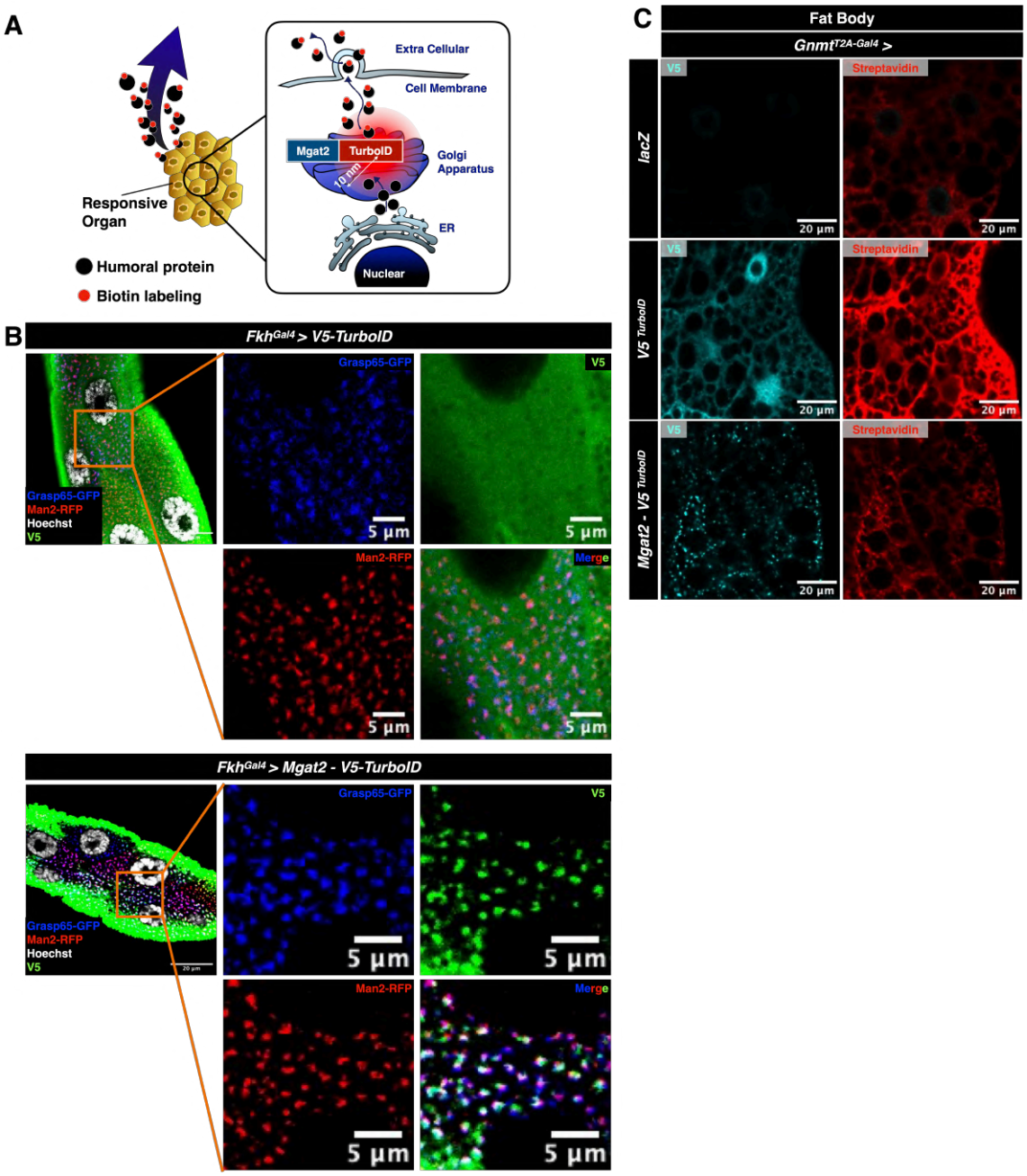

Figure 2　Subcellular localization and biotin-labeling of Mgat2-TurboID.

(A) Schematic view of the secretome analysis. Tissue specific expression of *Mgat2-TurboID* enable comprehensive biotin-labeling of secretory pathway proteins via nearby labeling in Golgi apparatus.

(B) Cellular localization of Mgat2-V5-TurboID and V5-TurboID. Over-expression of each genotype in the salivary gland with *Fkh*^{Gal4}. V5 signal (Green) shows localization of each construct. Grasp65 (Blue) is a marker of Cis-Golgi, and Man2 (Red) is a marker of Medial-Golgi. White scale bar, 5 μm or 20 μm.

(C) Subcellular localization and biotin-labeling of Mgat2-V5-TurboID and V5-TurboID. Over-expression of each genotype in the FB with *Gnmt*$^{T2A\text{-}Gal4}$. V5 signal (Cyan) and biotin-labeling (Red) was stained with anti-V5 antibody and Streptavidin-Cy3 respectively. White scale bar, 20 μm.

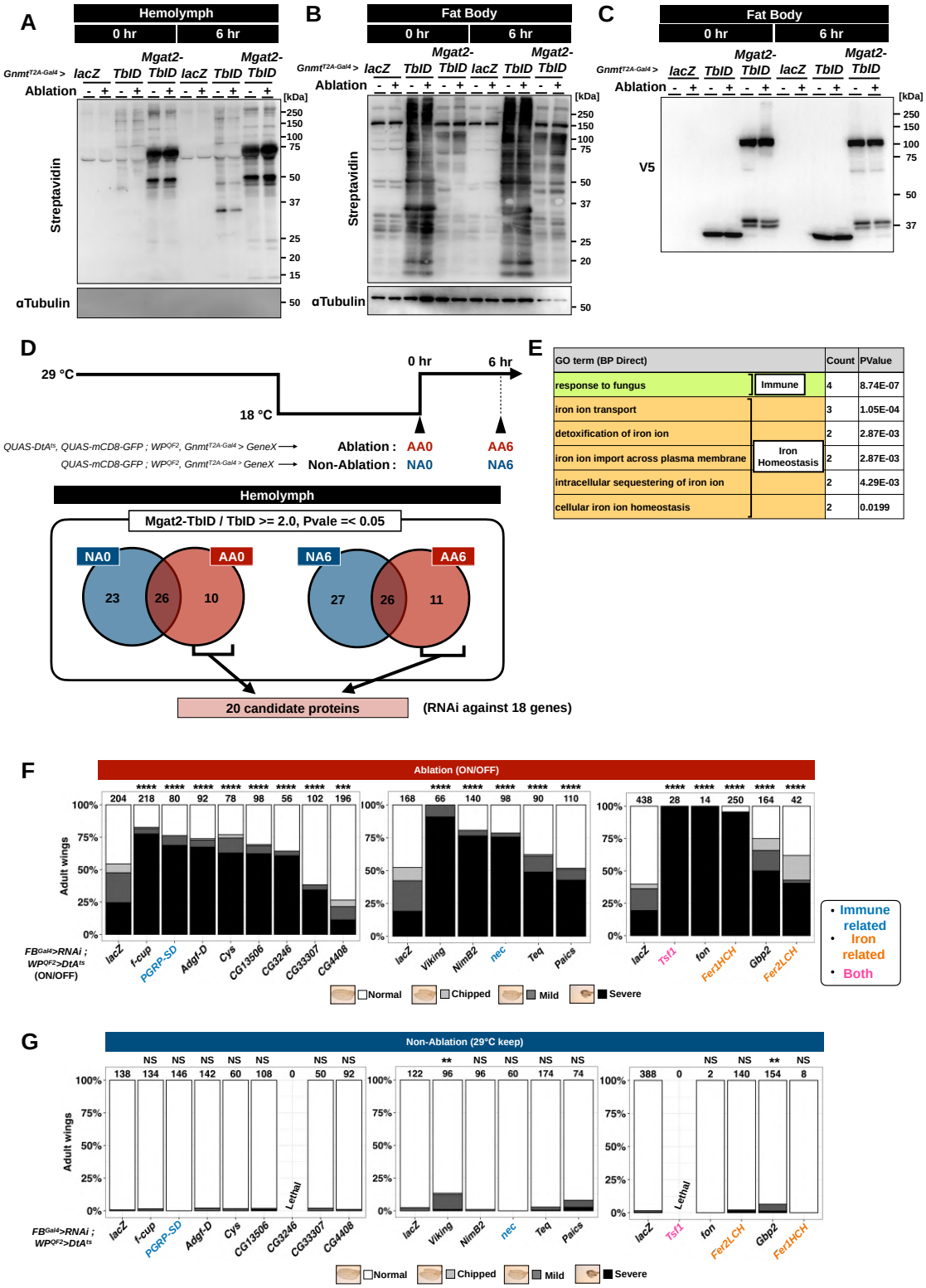

Figure 3 Secretome analysis using Mgat2-TurboID.

(A and B) Western blotting for biotinylated proteins (detected by Streptavidin-HRP) in the hemolymph (A) and the FB (B). *LacZ* (Control without TurboID), *TurboID* (TbID) and

Mgat2-TurboID (Mgat2-TbID) were expressed in the FB with $Gnmt^{T2A\text{-}Gal4}$. Samples were collected both in the Ablation and Non-Ablation and in each time points of 0 hr and 6 hr. α Tubulin were used as internal control.

(C) Western blotting for V5-tagged proteins in the FB. V5-TurboID and Mgat2-V5-TurboID were estimated to be 36.2 kDa and 92.5 kDa respectively.

(D) Scheme for temperature change and sampling. Analysis of biotinylated proteins in the hemolymph and FB at two time points. Under each condition, proteins detected with *Mgat2-TurboID* at more than twice the amount detected with *V5-TurboID* ($p < 0.05$) were extracted.

(E) GO analysis of 20 proteins in (D) with DAVID.

(F and G) RNAi screening of candidate proteins in Ablation (F) and Non-Ablation (G). Immune related proteins are indicated in blue, iron related proteins are indicated in orange, and proteins involved in both are indicated with magenta. Statistical analysis was conducted using the Fisher's exact test to compare the control ($WP^{QF2}>DtA^{ts}$; $FB^{Gal4}>lacZ^{RNAi}$) with the treated larvae. NS: not significant, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.

Supporting Information

This article contains supporting information (41-43).

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Author contributions

Y. Y., S. K., and M. M. designed the experiments and wrote the manuscript. S. K. and M. M. supervised the study. Y.Y. performed the experiments and analyzed the data.

Declarations

The authors declare no competing or financial interests.

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