1. 特許取得

該当なし

2. 実用新案登録

該当なし

3.その他

該当なし

研究協力者

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分担研究報告書

マウスにおける環境因子曝露とFUT8活性の肺気腫変化に及ぼす影響の解析

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研究要旨　本研究事業は肺気腫の病態とその成因を糖鎖異常によるシグナル伝達経路の変化を通して解析する事により、肺気腫に対する治療戦略を提供することを目的として開始された。肺気腫の主要な原因である喫煙によるFUT8活性の変化および肺気腫との関係をマウスへの喫煙実験を用いて解析した。1日4本、3ヶ月間の喫煙曝露により、Fut8 ヘテロ型ノックアウトマウス(Fut8+/-)では野生型マウスと比較して有意な肺胞マクロファージの増加を認めた。FUT8 活性は、野生型および Fut8+/-いずれも、喫煙後に有意な活性の低下を認めた。さらに、肺気腫の指標である平均肺胞径は、Fut8+/-で喫煙後に有意に増加を示した。従来、喫煙曝露による肺気腫のモデルマウスの作成には6ヶ月必要と考えられてきたため、Fut8+/-への喫煙曝露モデルは、早期肺気腫発症モデルと位置付けることが可能であり、新規COPD治療候補薬の評価などにおいて、大変有意義なモデルと考えられる。

A. 研究目的

慢性閉塞性肺疾患(COPD)はタバコなどの有毒な粒子やガスの吸入によって生じた、肺の炎症反応に基づく進行性の気流制限を呈する疾患である。現在、世界の死亡原因の第4位となっており、本疾患は世界的に重大な問題である。日本においても 40 歳以上の男性の 16.4%、女性の 5.0%、全体の8.6%という高い有病率(530万人)であることが明らかになってきた。

現在の COPD の治療は、禁煙・気管支拡張剤の投与が中心である。病因としては、1)プロテアーゼ・アンチプロテアーゼ不均衡、2)オキシダント・アンチオキシダント不均衡などが考えられその意義が検討されてきたが、プロテアーゼやオキシダントをターゲットとした治療法は確立されておらず、新たな機序で COPD の病態を抑制できる治療薬の開発が強く求められている。

糖鎖の機能解析の研究において、N 型糖鎖にコアフコースが付加(α1, 6 フコシル化)されると、そのターゲットタンパク質の機能が変化すること、またコアフコシル化を触媒する Fut8(α1,6 fucosyltransferase)の遺伝子欠損マウス(ノックアウトマウス)は、著明な成長障害と肺気腫様病変を示すことを明らかにしてきた。

この肺気腫様病変は、TGFβ受容体に対するコアフコースの付加がなされないため、TGFβ受容体の下流シグナルが減弱し、抑制系に働く Smad のリン酸化が十分に起こら

なくなることから細胞外マトリックスの分解を主につかさどるマトリックスメタロプロテアーゼ(MMP)の遺伝子発現が高まり、肺胞壁の合成と分解のバランスが崩れることにより肺気腫様の病変が引き起こされることを明らかにした。この研究により、糖鎖異常が肺気腫の原因になる可能性を示した。

今回は、野生型とほぼ同じ生存を示し、FUT8 活性が野生型に比較して約半分に減弱しているヘテロ型のノックアウトマウス(Fut8+/-)を用いて喫煙曝露を行い、FUT8 活性の変化および肺気腫との関連について検討することを目的とした。

B. 研究方法

マウスにおけるタバコ曝露とFUT8活性の変化の肺気腫に及ぼす影響の解析の検討:

肺気腫発症の最も重要な原因である喫煙が肺気腫の発症・進展に及ぼす分子メカニズムは十分に解明されてはいない。喫煙により糖鎖構造に変化が起きることが新たな発症原因となる可能性を探るため、マウスに対する喫煙実験を行い、FUT8 活性の変化が、炎症細胞浸潤や肺気腫の発症に作用するかどうかを、気管支肺胞洗浄・平均肺胞径測定を行いて検討する。

Fut8 のホモ型ノックアウトマウスは生後まもなく死亡してしまうため、安定して喫煙実験に使用することができないが、ヘテロ型のノックアウトマウス(Fut8+/-)は、野生型とほぼ同じ生存を示し、さらに FUT8 活性が野生型に比較して約半分に減弱していることが知られている。このヘテロ型ノックアウトマウスを用いて喫煙曝露を行った。喫煙曝露は、1日4本、週6回間施行した。タバコはケンタッキー大学のResearch Cigarette(2R4F)を使用した。

(倫理面への配慮)

動物実験に関しては、「生労働省の所管する実施機関における動物実験等の実施に関する基本指針」 ならびに「動物実験の適正な実施に向けたガイドライン」を尊守して作成された実験計画により実施している。

C. 研究結果

1日4本、週6回、3ヶ月間の喫煙曝露を行い、炎症細胞浸潤の評価・FUT8 活性の変化・平均肺胞径の変化を検討した。1. 炎症細胞浸潤の評価

喫煙終了翌日に、気管支肺胞洗浄を行い、Diff Quickにて染色し、炎症細胞の浸潤の程度を比較検討した。肺胞マクロファージ数は、野生型・Fut8+/-いずれにおいても、喫煙後に有意に増加した。さらに、喫煙後の Fut8+/-における肺胞マクロファージ数の増加は野生型と比較しても優位な上昇であった(図1)。

2. 喫煙による FUT8 活性の変化

喫煙終了翌日に、左肺を摘出し、FUT8 活性を測定した。FUT8 活性は、野生型・Fut8+/-いずれにおいても、喫煙後に有意に減少した(図2)。

3. 平均肺胞径の測定

喫煙終了翌日に、右肺を25cmH2O 圧にて10分間ホルマリン固定した。固定した肺は、24時間の固定の後、包埋しH.E.標本を作製した。この H.E.標本を用いて、肺胞径を測定し、平均肺胞径を算出した。摘出し、FUT8 活性を測定した。平均肺胞径は、野生型では 33.42±2.9 から 36.58±3.77 へ変化し、有意な増加は認めなかった。Fut8+/-においては、34.46±2.77 から 40.86±1.26 へと増加し、有意な増加を認めた(図3)。

D. 考察

従来、喫煙曝露による肺気腫のモデルマウスの作成には6ヶ月間を要すると考えられてきた。今回の我々の検討により、Fut8+/-への喫煙曝露モデルは、3ヶ月という従来よりも短縮した期間で肺気腫を作成できる、早期肺気腫発症モデルと位置付けることができる。

これまで、COPD の病因としては、プロテアーゼ・アンチプロテアーゼ不均衡やオキシダント・アンチオキシダント不均衡などが考えられてきたが、十分な治療法がない現状としては、新たな機序で COPD の病態を抑制できる治療薬の開発が強く求められている.

今回の我々の結果により、FUT8 活性の低下が、肺気腫の発症に関与していると考えられるが、この FUT8 活性の変化が、どのように肺気腫を引き起こすか、既存のプロテアーゼ・アンチプロテアーゼ不均衡やオキシダント・アンチオキシダント不均衡への影響の有無、アポトーシス誘導の有無、再生への影響など、その分子機序の解明が今後の課題となる。

E. 結論

Fut8+/-への喫煙曝露モデルは、3ヶ月という従来よりも短縮した期間で肺気腫を作成できる、早期肺気腫発症モデルと位置付けることができる。

新たな機序で COPD の病態を抑制できる治療薬の開発が強く求められている現状のなかで、この Fut8+/-への喫煙曝露モデルは、今後の COPD 治療候補薬の評価などにおいて、大変有意義なモデルと考えられる。

G. 研究発表

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該当なし

2. 学会発表

該当なし

H. 知的財産権の出願・登録状況

(予定を含む。)

1. 特許取得

該当なし

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該当なし

3.その他

該当なし

図1

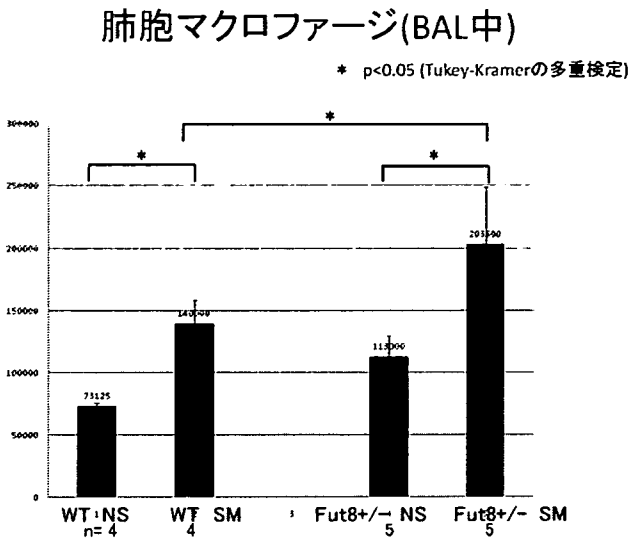

肺胞マクロファージ(BAL中)
* p<0.05 (Tukey-Kramerの多重検定)
*
*
*
73125
WT NS
n= 4
WT SM
4
Fut8+/− NS
5
Fut8+/− SM
5

図2

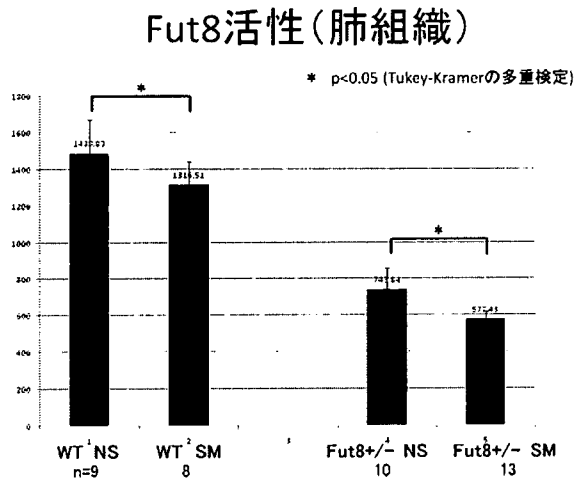
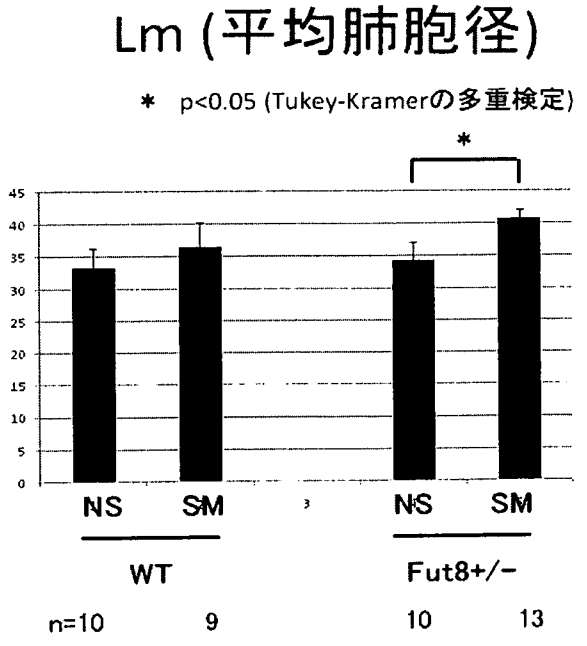

Fut8活性(肺組織)
* p<0.05 (Tukey-Kramerの多重検定)
*
*
WT NS
n=9
WT SM
8
Fut8+/− NS
10
Fut8+/− SM
13

図3


Lm (平均肺胞径)
* p<0.05 (Tukey-Kramerの多重検定)
*
NS
SM
NS
SM
WT
Fut8+/−
n=10
9
10
13

研究成果の刊行に関する一覧表

著書

無し

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REVIEW ARTICLE

Branched N-glycans regulate the biological functions of integrins and cadherins

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Glycosylation is one of the most common post-translational modifications, and approximately 50% of all proteins are presumed to be glycosylated in eukaryotes. Branched N-glycans, such as bisecting GlcNAc, β-1,6-GlcNAc and core fucose (α-1,6-fucose), are enzymatic products of *N*-acetylglucosaminyltransferase III, *N*-acetylglucosaminyltransferase V and α-1,6-fucosyltransferase, respectively. These branched structures are highly associated with various biological functions of cell adhesion molecules, including cell adhesion and cancer metastasis. E-cadherin and integrins, bearing N-glycans, are representative adhesion molecules. Typically, both are glycosylated by *N*-acetylglucosaminyltransferase III, which inhibits cell migration. In contrast, integrins glycosylated by *N*-acetylglucosaminyltransferase V promote cell migration. Core fucosylation is essential for integrin-mediated cell migration and signal transduction. Collectively, N-glycans on adhesion molecules, especially those on E-cadherin and integrins, play key roles in cell–cell and cell–extracellular matrix interactions, thereby affecting cancer metastasis.

Introduction

Glycosylation is involved in a variety of physiological and pathological events, including cell growth, migration, differentiation and tumor invasion. It is well known that approximately 50% of all proteins are glycosylated [1]. Glycosylation reactions are catalyzed by the action of glycosyltransferases, which add sugar chains to various complex carbohydrates, such as glycoproteins, glycolipids, and proteoglycans. Functional glycomics, which uses sugar remodeling by glycosyltransferases, is a promising tool for the characterization of glycan functions [2]. A large number of glycosyltransferases (products of approximately 170 genes) have been cloned [3,4], and some of their important functions have been clarified [5,6]. In this review,

Abbreviations
ADCC, antibody-dependent cellular cytotoxicity; ECM, extracellular matrix; EGFR, epithelial growth factor receptor; FAK, focal adhesion kinase; Fut8, α-1,6-fucosyltransferase; GnT, *N*-acetylglucosaminyltransferase; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β.

the specific biological functions of major glycosyltransferases involved in N-glycan biosynthesis, such as *N*-acetylglucosaminyltransferase (GnT) III [7,8], GnT-V [9–11], and α-1,6-fucosyltransferase (Fut8) [12–14], are discussed, thereby demonstrating the importance of glycosyltransferase regulation to the function of the adhesion molecules integrin and E-cadherin.

Biological significance of GnT-III, GnT-V and Fut8

GnT-III

GnT-III catalyzes the addition of GlcNAc to mannose that is β-1,4-linked to an underlying *N*-acetylglucosamine, producing what is known as a 'bisecting' GlcNAc linkage. GnT-III is ubiquitous in all tissues, although relatively higher GnT-III activity is found in kidney and brain [15]. GnT-III is generally regarded as a key glycosyltransferase in N-glycan biosynthetic pathways, and contributes to the inhibition of metastasis (Fig. 1). The introduction of a bisecting GlcNAc catalyzed by GnT-III suppresses additional processing and elongation of N-glycans. These reactions, which are catalyzed *in vitro* by other glycosyltransferases, such as GnT-IV, GnT-V, and GnT-VI, do not proceed, because the enzymes cannot utilize the bisected oligosaccharide as a substrate [16]. When the GnT-III gene was transfected into melanoma B16 cells with high metastatic potential, the sugar chains on the cell surface were remodeled. A lung metastasis assay was performed by injecting B16 cells into syngeneic mice via the tail vein. Interestingly, the lung metastatic foci were significantly suppressed in the mice injected with GnT-III-transfected melanoma B16 cells as compared with mice treated with mock-transfected cells [17]. GnT-III also contributes to suppression of metastasis by remodeling some important glycoproteins, such as epithelial growth factor receptor (EGFR) [18–20], and adhesion molecules such as integrin and cadherin, as described below. GnT-III also inhibits the formation of the α-Gal epitope, which is a major xenotransplantation antigen that is problematic in swine-to-human organ transplantation [21]. Moreover, GnT-III affects antibody-dependent cellular cytotoxicity (ADCC) activity [22], although, the effect of GnT-III on ADCC activity appears to be less than that of core fucose structures, as described below.

Transgenic mice, in which GnT-III was expressed specifically in the liver by use of a serum amyloid P component gene promoter, exhibited fatty liver. It has been proposed that ectopic expression of GnT-III disrupts the function of apolipoprotein B, resulting in abnormal lipid accumulation [23]. To explore the physiological roles of GnT-III, GnT-III-deficient mice have been established using gene targeting. These mice are viable and reproduce normally, suggesting that GnT-III and the bisected N-glycans apparently are not essential for normal development [24]. Because no physical abnormalities were apparent, the physiological roles of GnT-III are yet to be identified.

GnT-V

In contrast to the functions of GnT-III, GnT-V catalyzes the formation of β-1,6-GlcNAc branching

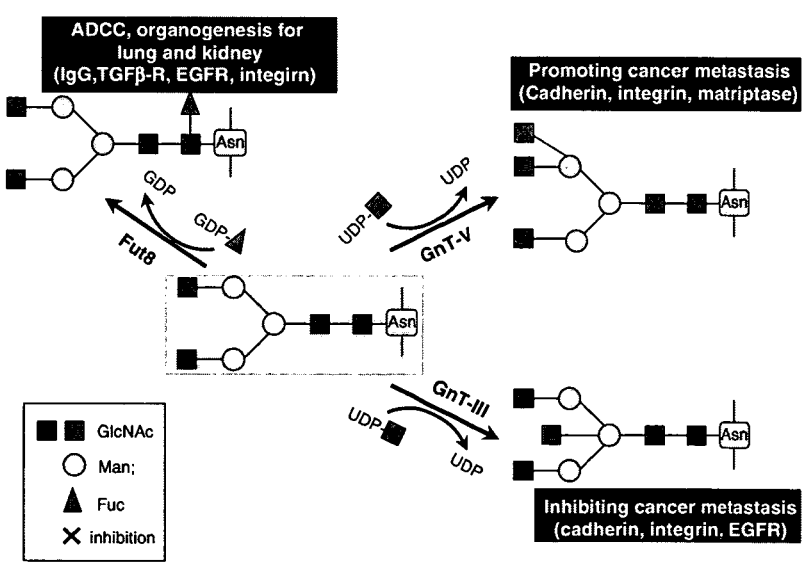

Fig. 1. Glycosylation reactions catalyzed by the glycosyltransferases GnT-III and GnT-V, as well as by Fut8, and their biological functions.

structures, which play important roles in tumor metastasis [25,26]. The activity of GnT-V is higher in the small intestine than in other normal tissues [15]. A relationship between GnT-V and cancer metastasis has been reported by Dennis *et al.* [27] and Yamashita *et al.* [28]. Studies of transplantable tumors in mice indicate that the product of GnT-V directly contributes to cancer growth and subsequent metastasis [29,30] (Fig. 1). In contrast, somatic tumor cell mutants that are deficient in GnT-V activity produce fewer spontaneous metastases and grow more slowly than wild-type cells [27,31]. Dennis *et al.* found that mice lacking glycosyltransferase GnT-V (encoded by *Mgat5*) cannot add β-1,6-GlcNAc to N-glycans, so the most complex types of N-glycans, such as tetra-antennary and poly(*N*-acetyllactosamine), cannot be formed. These mice failed to develop normally and displayed a variety of phenotypes associated with altered susceptibility to autoimmune diseases, enhanced delayed-type hypersensitivity, and lowered T-cell activation thresholds, due to direct enhancement of T-cell receptor clustering [30,32]. The authors proposed that modification of growth factor receptors, such as receptors for epithelial growth factor, insulin-like growth factor, platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), with N-glycans using poly(*N*-acetyllactosamine) would cause preferential receptor binding to galectins, resulting in formation of a lattice that opposes constitutive endocytosis. As a result, intracellular signaling and, consequently, cell migration and tumor metastasis would be enhanced [33]. Very recently, the same group used both computational modeling and experimental data obtained from studies of T lymphocytes and epithelial cells to show that galectin binding to N-glycans on membrane glycoproteins enhances surface residency, and is dependent on N-glycan number (protein encoded) and N-glycan GlcNAc-branching activity, which, in turn, is dependent on UDP-GlcNAc availability. Receptor kinases that promote growth have more potential N-glycan addition sites than receptor kinases that halt growth and initiate differentiation. Thus, glycoproteins with many N-glycan molecules, such as epithelial growth factor receptor (EGFR), insulin-like growth factor receptor, fibroblast growth factor receptor, and PDGF receptor, exhibit superior galectin binding and early, graded increases in cell surface expression in response to increasing UDP-GlcNAc concentrations (i.e. supply to Golgi GlcNAc branching). In contrast, glycoproteins with one or only a few N-glycans (e.g. TGF-β receptor, CTLA-4, and GLUT4) exhibit delayed, switch-like responses. This result suggests that N-glycan branching might act as a metabolic sensor for the balance of cell growth and arrest signals [34].

Moreover, hepatic GnT-V upregulation in a rodent model of hepatocarcinogenesis and liver regeneration has been reported [35]. Matriptase, a serine proteinase, in the GnT-V transfectant was resistant to autodigestion and to exogenous trypsin. This resistance may lead to constitutively active matriptase, which is highly associated with cancer invasion and metastasis, because matriptase activates the precursor of hepatocyte growth factor precursor by proteolytic digestion. In GnT-V transgenic mice, matriptase was shown to cause cancer invasion and metastasis [36,37]. Taken together, these findings suggest that inhibition of GnT-V might be useful in the treatment of malignancies by interfering with the metastatic process.

Fut8

Fut8 catalyzes the transfer of a fucose residue from GDP-fucose to position 6 on the innermost GlcNAc residue of hybrid and complex N-linked oligosaccharides on glycoproteins, resulting in core fucosylation (α-1,6-fucosylation) (Fig. 1). Fut8 activity in brain is higher than in other normal tissues [12]. Fut8 is the only core fucosyltransferase found in mammals, but there are core α-1,3-fucose residues in plants, insects, and probably other species as well.

Core fucosylated glycoproteins are widely distributed in mammalian tissues, and may be altered under pathological conditions, such as hepatocellular carcinoma and liver cirrhosis [38,39]. High Fut8 expression was observed in a third of papillary carcinomas and was directly linked to tumor size and lymph node metastasis. Thus, Fut8 expression may be a key factor in the progression of thyroid papillary carcinomas [40]. It has also been reported that deletion of core fucose from the IgG_1 molecule enhances ADCC activity by as much as 50–100-fold. This result indicates that core fucose is an important sugar chain in terms of ADCC activity [41]. Recently, the physiological functions of core fucose have been investigated in core fucose-deficient mice [42]. *Fut8*-knockout (*Fut8*$^{-/-}$) mice showed severe growth retardation, and 70% died within 3 days after birth. The surviving mice suffered from emphysema-like changes in the lung that appear to be due to a lack of core fucosylation of the TGF-β1 receptor, which consequently results in marked dysregulation of TGF-β1 receptor activation and signaling. Loss of core fucosylation also resulted in downregulation of the EGFR-mediated signaling pathway [43]. Downregulation of TGF-β receptor, EGFR and PDGF receptor activation is a plausible explanation for the

emphysema-like changes and growth retardation [43–45]. Taken together, these results suggest that core fucose modification of functional proteins affects important physiological functions.

Important adhesion molecules expressed on the cell surface

Integrin

Integrins comprise a family of receptors that are important for cell adhesion. Integrins consist of α- and β-subunits. Each subunit has a large extracellular region, a single transmembrane domain, and a short cytoplasmic tail (except for β_4). The N-terminal domains of the α- and β-subunits associate to form the integrin headpiece, which contains the extracellular matrix (ECM) binding site. The C-terminal segments traverse the plasma membrane and mediate interactions with the cytoskeleton and with signaling molecules. On the basis of extensive searches of the human and mouse genomic sequences, it is now known that 18 α-subunits and eight β-subunits assemble into 24 integrins. Among these integrins, 12 members that contain the β_1-subunit have been identified. Each of these integrins appears to have a specific and nonredundant function. Gene knockouts of the α- and βsubunits have been created. Each knockout has a distinct phenotype, reflecting the different roles of the various integrins [44]. For example, the α_3-knockout mouse has impaired development of the lung and kidney [46].

Integrin engagement during cell adhesion leads to intracellular phosphorylation, such as phosphorylation of focal adhesion kinase (FAK), thereby regulating gene expression, cell growth, cell differentiation and survival from apoptosis [47]. These events are controlled by biochemical signals generated by ligand-occupied and clustered integrins. Recent studies have also shown that growth factor-induced proliferation, cell cycle progression and cell differentiation require cellular adhesion to the ECM, a process that is mediated by integrins [48,49]. Therefore, integrins are adhesion molecules that transmit information across the plasma membrane in both directions.

E-cadherin

The cadherins comprise another important family of adhesion molecules that function in cell recognition, tissue morphogenesis, and tumor suppression [50]. E-cadherin is the prototypical member of these calcium-dependent cell adhesion molecules and mediates homophilic cell–cell adhesion. Loss of E-cadherin expression or function in epithelial carcinoma cells has long been considered to be a primary cause of disruption of tight epithelial cell–cell contacts and release of invasive tumor cells from the primary tumor [51]. E-cadherin is a widely acting suppressor of epithelial cancer invasion and growth, and its functional elimination represents a key step in the acquisition of the invasive phenotype for many tumors. E-cadherin is found in epithelia, where the adhesion molecule promotes tight cell–cell associations, known as adherens junctions. In contrast, N-cadherin is found primarily in neural tissues and fibroblasts, where it is thought to mediate a less stable and more dynamic form of cell–cell adhesion [52]. Therefore, cell–cell adhesion is believed to be both temporally and spatially regulated during development.

Sugar remodeling regulates integrin and E-cadherin function

Integrin sugar chains play important roles in the biological functions of integrins

A growing body of evidence indicates that the presence of the appropriate oligosaccharide can modulate integrin activation [53]. When human fibroblasts were cultured in the presence of l-deoxymannojirimycin, an inhibitor of α-mannosidase II that prevents N-linked oligosaccharide processing, immature $\alpha_5\beta_1$ appeared on the cell surface, and fibronectin-dependent adhesion was greatly reduced. Treatment of purified $\alpha_5\beta_1$ with *N*-glycosidase F, also known as PNGase F, which cleaves between the innermost GlcNAc and asparagine N-glycan residues from N-linked glycoproteins, blocked $\alpha_5\beta_1$ binding to fibronectin and prevented the inherent association between subunits [54]. This result suggests that N-glycosylation is essential for functional $\alpha_5\beta_1$. Recently, it was found that N-glycans on the β-propeller domain of the α_5-subunit are essential for $\alpha_5\beta_1$ heterodimerization, cell surface expression, and biological function [55]. Altered expression of the N-glycans in $\alpha_5\beta_1$ might contribute to the adhesive properties of tumor cells and to tumor formation. When NIH3T3 cells were transformed with the *Ras* oncogene, cell spreading on fibronectin was greatly enhanced, due to an increase in β-1,6-GlcNAc branched tri-antennary and tetra-antennary oligosaccharides in $\alpha_5\beta_1$ [56]. Similarly, characterization of the carbohydrate moieties in $\alpha_3\beta_1$ from nonmetastatic and metastatic human melanoma cell lines showed that expression of β-1,6-GlcNAc branched structures was higher in metastatic cells than in nonmetastatic cells, confirming the notion that the β-1,6-GlcNAc branched structure confers invasive and metastatic properties to

cancer cells. Integrin surface expression and activation appear to be dependent on branched N-glycans, and an important aspect of this dependence is galectin binding. It is worth noting that fibronectin polymerization and tumor cell motility are regulated by binding of galectin-3 to branched N-glycan ligands that stimulate focal adhesion remodeling, FAK and phosphoinositide 3-kinase (PI3K) activation, local F-actin instability, and $\alpha_5\beta_1$ translocation to fibrillar adhesions [57].

Furthermore, when exploring possible mechanisms for the increase in β-1,6-branched N-glycans on the surface of metastatic cancer cells, Guo *et al.* found that both cell migration towards fibronectin and invasion through Matrigel were significantly stimulated in GnT-V-transfected cells [58]. Increased numbers of branched sugar chains inhibited $\alpha_5\beta_1$ clustering and organization of F-actin into extended microfilaments in cells plated on fibronectin-coated plates. This observation confirms the hypothesis that the degree of cellular adhesion to the ECM substrate is a critical factor in the regulation of the cell migration rate [59]. Conversely, deletion of GnT-V modification in mouse embryonic fibroblasts resulted in enhanced integrin clustering and activation of $\alpha_5\beta_1$ transcription by protein kinase C signaling, which, in turn, upregulated cell surface expression of $\alpha_5\beta_1$, resulting in increased matrix adhesion and decreased migration [60].

Interestingly, overexpression of GnT-III inhibited $\alpha_5\beta_1$-mediated cell spreading and migration, and phosphorylation of FAK [61]. The binding affinity of $\alpha_5\beta_1$ for fibronectin was significantly reduced after introduction of a bisecting GlcNAc into the α_5-subunit.

Introduction of GnT-III reduces metastatic potential, whereas the product of GnT-V, β-1,6-GlcNAc branched N-glycan, contributes to cancer progression and metastasis [27]. The reaction that is catalyzed by GnT-V is inhibited by GnT-III, as shown by *in vitro* substrate specificity studies, as described above [16]. The hypothesis that competition between GnT-III and GnT-V affects cell migration and tumor metastasis has not been verified directly. Recently, it was reported that $\alpha_3\beta_1$, which is highly associated with tumor metastasis, can be modified by either GnT-III or GnT-V (Fig. 2). This finding shows that GnT-III inhibits GnT-V-stimulated $\alpha_3\beta_1$-mediated cell migration. The priority of GnT-III for modification of the α_3-subunit may explain inhibition of GnT-V-induced cell migration by GnT-III [62]. These results were the first to demonstrate that GnT-III and GnT-V competitively modify the same target glycoprotein and that this competition between enzymes either positively or negatively regulates the biological function of the target protein. Furthermore, these results suggest that competition between enzymes occurs not only *in vitro*, but also in living cells, and might provide new insights into the molecular mechanism of tumor metastasis (Fig. 3).

However, the effects of GnT-III and its products on cancer progression are equivocal. Stanley *et al.* reported that progression of hepatic neoplasms induced by diethylnitrosamine injection and subsequent treatment with phenobarbitol was severely retarded in GnT-III-knockout mice, suggesting that bisecting GlcNAc facilitates tumor progression in liver [63]. This discrepancy has not been well examined, but it would be interesting to study whether GnT-III and

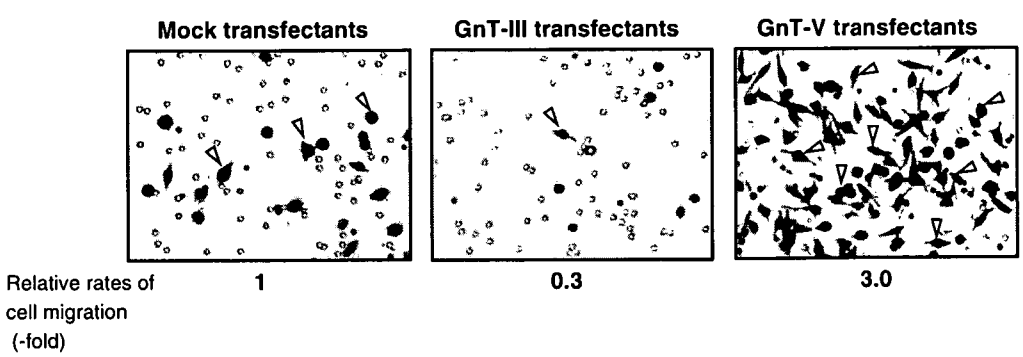

Fig. 2. Decreased and increased cell migration of MKN45 cells (human gastric cancer cell line) on laminin 5 induced by GnT-III and GnT-V, respectively. Cell migration was determined using the Transwell assay as described previously [62]. Arrows indicate the migrated cells. Briefly, Transwells (BD Bioscience, Franklin Lakes, NJ) were coated with 5 nM recombinant LN5 in NaCl/P_i by an overnight incubation at 4 °C. Serum-starved cells (2×10^5 per well) in 500 μL of 5% fetal bovine serum medium were seeded in the upper chamber of the plates. After incubation overnight at 37 °C, cells in the upper chamber of the filter were removed with a wet cotton swab. Cells on the lower portion of the filter were fixed and stained with 0.5% crystal violet. Each experiment was performed in triplicate, and three randomly selected microscopic fields within each well were counted. Figure partly reproduced and modified from the authors' original work [62].

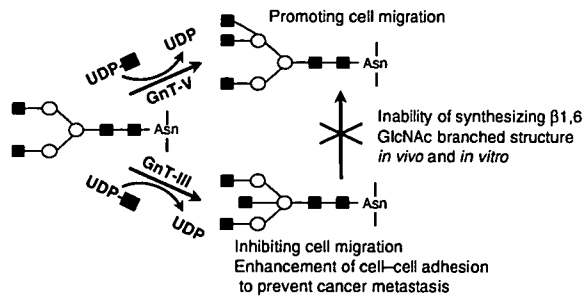

Fig. 3. Introduction of bisecting GlcNAc suppressed β-1,6-GlcNAc branch formation on $\alpha_3\beta_1$. It is well known that GnT-V cannot use the bisected oligosaccharide, a product of GnT-III, as a substrate *in vitro* [16,74]. Therefore, it has been postulated that cancer metastasis induced by GnT-V can be blocked by GnT-III overexpression, due to substrate competition for the same sugar chain. This hypothesis was confirmed by an *in vivo* study [62]. The products of GnT-V on $\alpha_3\beta_1$ promoted cell migration, whereas expression of GnT-III suppressed GnT-V-induced cell migration and products.

bisecting GlcNAc affect tumor metastasis in an experimental system other than knockout mice.

In addition, it was recently reported that overexpression of GnT-III in Neuro2a cells enhanced neurite outgrowth under serum deprivation conditions [64]. The results of this study clearly demonstrated the importance of bisecting GlcNAc N-glycans introduced by GnT-III in Neuro2a cell differentiation. Overexpression of GnT-III in the cells induced axon-like processes with numerous neurites and swellings, in which β_1 was localized, under conditions of serum deprivation. Enhanced neuritogenesis was suppressed by addition of either a bisecting GlcNAc-containing N-glycan or E_4-phytohemagglutinin, which preferentially recognizes bisecting GlcNAc. GnT-III-promoted neuritogenesis was also significantly perturbed by treatment with a functional blocking antibody to β_1. These findings may explain why bisecting GlcNAc-containing N-glycans are abundant in the brain [65]. In fact, mice carrying an inactive GnT-III mutant have an atypical neurological phenotype [66]. The data obtained in these studies suggest new roles for GnT-III and integrins in neuritogenesis.

On the other hand, the role of core fucosylation in $\alpha_3\beta_1$-mediated events has been studied using *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$embryonic fibroblasts [67]. $\alpha_3\beta_1$-mediated migration was reduced in *Fut8*$^{-/-}$cells. Moreover, integrin-mediated cell signaling was reduced in *Fut8*$^{-/-}$ cells. Reintroduction of *Fut8* has the potential to reverse such impairments (Fig. 4). Collectively, these results suggest that core fucosylation is essential for functional $\alpha_3\beta_1$. Although integrins have multiple potential N-glycosylation sites, only N-glycans located on certain motifs regulate integrin conformation and biological function. For example, only N-glycans located on either the β-propeller of α_5 [55] or the I-like domain of β_1 or β_3 [68] contribute to the regulation of integrin function. Therefore, we speculate that modification of particular sites, which are involved in regulation of the conformation of integrin, determine the extent of cell migration.

The mutual regulation of GnT-III and E-cadherin

To a certain degree, mutual regulation of GnT-III expression and E-cadherin-mediated cell–cell interaction exists as a positive feedback loop. Overexpression of GnT-III increased E-cadherin-mediated homotypic adhesion and suppressed phosphorylation of the E-cadherin–β-catenin complex during cell–cell adhesion

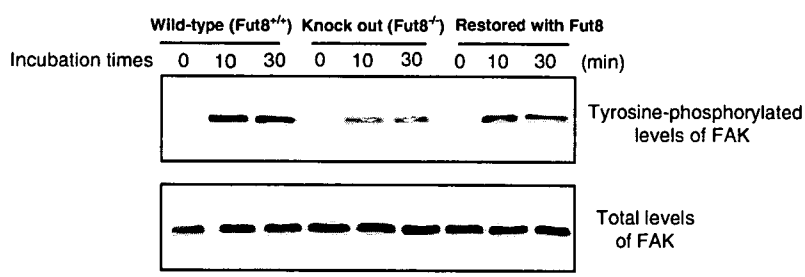

Fig. 4. Integrin-stimulated phosphorylation of FAK was reduced in *Fut8*$^{-/-}$ cells. Serum-starved *Fut8*$^{+/+}$, *Fut8*$^{-/-}$ mouse embryonic fibroblasts and restored cells were respectively detached and held in suspension for 60 min to reduce the detachment-induced activation. Cells were then replated on dishes coated with LN5 (5 nM) for the indicated times. The cell lysates were blotted with antibody against phosphotyrosine FAK (pY397) (BD). Equal loading was confirmed by blotting with an antibody against total FAK (BD), as described previously [67]. $\alpha_3\beta_1$-stimulated tyrosine phosphorylation of FAK was reduced in *Fut8*$^{-/-}$ cells as compared with *Fut8*$^{+/+}$ cells. Moreover, downregulation of phosphorylation in *Fut8*$^{-/-}$ cells was restored in the rescued cells, suggesting that lack of core fucosylation negatively regulated the $\alpha_3\beta_1$-mediated signaling pathway. Figure partly reproduced and modified from the authors' original work [67].

[69,70]. E-cadherin, when located on the cell surface, is resistant to proteolysis. Overexpression of GnT-III results in retention of E-cadherin at the cell border. The increased GnT-III product on E-cadherin reduces phosphorylation of β-catenin either by EGFR or by Src signaling. As a result, β-catenin remains tightly complexed with E-cadherin and is not translocated into the nuclei. Otherwise, β-catenin enhances expression of genes that promote cell growth or oncogenesis. Conversely, GnT-III is regulated by E-cadherin-mediated cell–cell adhesion [71]. GnT-III activity was increased under dense culture conditions as compared with sparse culture conditions. Regulation of cadherin-mediated adhesion and the associated adherens junctions is thought to control the dynamics of adhesive interactions between cells during tissue development and homeostasis, as well as during tumor cell progression. In fact, E-cadherin expression is highly regulated by epithelial cell–cell interactions [72]. However, significant regulation of GnT-III expression was observed only in epithelial cells that express basal levels of E-cadherin and GnT-III. However, GnT-III expression was not regulated in various cell types, as follows: MDA-MB231 cells, an E-cadherin-deficient cell line; MDCK cells, in which GnT-III expression is undetectable; and fibroblasts, which lack E-cadherin. To a certain extent, cells cultured under sparse and dense culture conditions can be viewed as cells in the proliferative and differentiative maintenance states, respectively. GnT-III expression was upregulated in cells cultured under dense conditions. In that study, GnT-III expression was significantly upregulated by cell–cell interactions. This would reasonably maintain cell differentiation rather than cell proliferation, as growth factor-mediated activation can be suppressed by the upregulation of GnT-III. In fact, the results of several studies suggest that E-cadherin can induce ligand-independent activation of EGFR and subsequent activation of Rac1 and MAP kinase, which appears to be involved in cell migration and proliferation [73]. Thus, it is possible that upregulation of GnT-III by cell–cell interaction might neutralize the signals responsible for maintenance of the cell differentiation phenotype, further supporting the notion that N-glycosylation plays an important role in cellular functions.

Future perspectives

It is well known that a large number of proteins undergo post-translational modification, which alters protein structure and function. Among the various post-translational modifications, glycosylation is not only the most common, but also the most important. As described above, modulation of adhesion molecule glycosylation might significantly alter the biological function of adhesion molecules. Because of the important roles of glycosylation, functional glycomics, which uses powerful methods of gene manipulation such as gene knockout and knockin, as well as small interfering RNA, and characterization of glycan structures using MS, will open new avenues for the study of physiological regulation of glycosylation of glycoproteins.

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The *O*-fucosyltransferase O-fut1 is an extracellular component that is essential for the constitutive endocytic trafficking of Notch in *Drosophila*

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Notch is a transmembrane receptor that mediates the cell-cell interactions necessary for many cell-fate decisions. Endocytic trafficking of Notch plays important roles in the activation and downregulation of this receptor. A *Drosophila O*-FucT-1 homolog, encoded by *O-fut1*, catalyzes the *O*-fucosylation of Notch, a modification essential for Notch signaling and ligand binding. It was recently proposed that O-fut1 acts as a chaperon for Notch in the endoplasmic reticulum and is required for Notch to exit the endoplasmic reticulum. Here, we report that O-fut1 has additional functions in the endocytic transportation of Notch. O-fut1 was indispensable for the constitutive transportation of Notch from the plasma membrane to the early endosome, which we show was independent of the *O*-fucosyltransferase activity of O-fut1. We also found that O-fut1 promoted the turnover of Notch, which consequently downregulated Notch signaling. O-fut1 formed a stable complex with the extracellular domain of Notch. In addition, O-fut1 protein added to conditioned medium and endocytosed was sufficient to rescue normal Notch transportation to the early endosome in O-fut1 knockdown cells. Thus, an extracellular interaction between Notch and O-fut1 is essential for the normal endocytic transportation of Notch. We propose that O-fut1 is the first example, except for ligands, of a molecule that is required extracellularly for receptor transportation by endocytosis.

KEY WORDS: Notch, *O*-fucosyltransferase, O-fut1, Endocytosis, *Drosophila*

INTRODUCTION

Notch signaling is an evolutionarily conserved mechanism that regulates a broad spectrum of cell-specification events through local cell-cell communication (Artavanis-Tsakonas et al., 1999). *Notch* encodes a single-pass transmembrane receptor protein with 36 epidermal growth factor-like (EGF) repeats and three Notch/LIN-12 repeats in its extracellular domain and six CDC10/Ankyrin repeats and a PEST-like sequence in its intracellular domain (Kidd et al., 1983; Wharton et al., 1985). In *Drosophila*, two ligands for Notch, Delta and Serrate, which are also transmembrane proteins, are known (Fleming, 1998). The binding between Notch and its ligands leads to a proteolytic cleavage within the transmembrane domain that releases the intracellular domain Notch^{ICD} (Mumm and Kopan, 2000). Notch^{ICD} then translocates to the nucleus and acts as a coactivator for a sequence-specific DNA-binding protein, Suppressor of Hairless, and this complex activates various target genes of the Notch signal (Bray and Furriols, 2001).

This main Notch pathway is evolutionarily conserved from nematodes to mammals (Lai, 2004). However, several additional processes, such as the intracellular transportation of Notch and its ligands, tightly regulate Notch signaling (Le Borgne et al., 2005). For example, the activation of Notch requires its endocytic incorporation and/or that of its ligand (Seugnet et al., 1997), and Notch endocytosis is also involved in the downregulation of Notch activity (Jékely and Rørth, 2003). Several regulators of the endocytic trafficking of Notch, such as the Nedd4 family proteins, Arrestin, Numb and Deltex, have been identified (Sakata et al., 2004; Wilkin et al., 2004; Mukherjee et al., 2005; Berdnik et al., 2002; Hori et al., 2004). In addition, recent findings show that mutations of genes that are generally involved in endocytosis affect the amount and activity of Notch (Giebel and Wodarz, 2006). Although the amount of Notch increased in these mutants, the Notch signal was inactivated in some of them and hyperactivated in others (Lu and Bilder, 2005; Vaccari and Bilder, 2005; Thompson et al., 2005; Moberg et al., 2005; Maitra et al., 2006; Herz et al., 2006). However, it is largely unknown how the endocytic pathway influences the activity of Notch.

In addition to its trafficking, the signaling activity of Notch is also influenced by its glycosylation. Analyses of the Notch signal in a mutant of the UDP-GlcNAc transporter gene, which is probably required for protein *N*-glycosylation, suggested that this modification is essential for the normal functioning of Notch (Goto et al., 2001; Selva et al., 2001). Furthermore, Notch undergoes *O*-linked fucosylation, and the functions of this modification have been studied extensively (Haines and Irvine, 2003). The EGF-like repeats of the Notch extracellular domain, which contain a consensus sequence, are modified by the *O*-linked tetrasaccharide Sia-α2,3-Gal-β1,4-GlcNAc-β1,3-Fuc (Moloney et al., 2000). A GDP-fucose protein *O*-fucosyltransferase1 catalyzes this *O*-linked fucosylation in mammals and *Drosophila* (Wang et al., 2001; Okajima and Irvine, 2002). In *Drosophila*, this enzyme is encoded by *O-fut1* (Okajima and Irvine, 2002). This *O*-fucosylation of Notch is essential for Notch signaling and Notch-ligand interactions (Okajima and Irvine, 2002; Sasamura et al., 2003; Okajima et al.,

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2003; Shi and Stanley, 2003). *N*-acetylglucosamine is subsequently added to this fucose moiety by a fucose-specific β1,3 *N*-acetylglucosaminyltransferase, Fringe (Moloney et al., 2000). This modification modulates Notch-ligand interactions (Panin et al., 1997; Brückner et al., 2000).

More recently, O-fut1 was shown to act as a chaperon for Notch, a function that does not require the *O*-fucosyltransferase enzymatic activity of O-fut1 (Okajima et al., 2005). Knocking down *O-fut1* by RNA interference (RNAi) in *Drosophila* cultured cells prevents the Notch extracellular domain polypeptide from being secreted into the medium (Okajima et al., 2005). When this Notch fragment and a mutant O-fut1 that lacks *O*-fucosyltransferase activity are co-expressed, the mutant O-fut1 still promotes the binding between the Notch polypeptide and its ligands (Okajima et al., 2005). These observations led to the proposal that O-fut1 is required for the proper folding of the Notch extracellular domain, and this function is independent of the enzymatic activity of O-fut1 (Okajima et al., 2005). Here, we demonstrated that O-fut1 has another, distinct, function in Notch signaling. Extracellular O-fut1 was required for the constitutive endocytic transportation of Notch to the early endosome, and this function was also independent of the *O*-fucosyltransferase activity of O-fut1.

MATERIALS AND METHODS

Fly stocks and genetics

We used *Canton-S* as the wild-type strain, *O-fut1*^{4R6} as the *O-fut1* mutant (Sasamura et al., 2003), *Delta*^{RevF10} *Serrate*^{RX106} as the double mutant of *Delta* and *Serrate* (Ligoxygakis et al., 1998) and *Notch*^{55e11} (Kidd et al., 1983) as the *Notch* mutant. *daughterless* (*da*)-*GAL4* (*GAL4*^{daG32}) (Wodarz et al., 1995), *engrailed* (*en*)-*GAL4* (Johnson et al., 1995) and *scalloped* (*sd*)-*GAL4* (Roy et al., 1997) were used as the GAL4 drivers. We used FLP/FRT (Xu and Rubin, 1993) and the MARCM (Lee and Luo, 1999) system to generate somatic mutant clones. To isolate the *Gmd* mutant, 171 independent *white* derivative strains were established from *GS13045* and screened using a genomic PCR method (Toba et al., 1999). To generate *O-fut1*$^{-}$ clones in the *Gmd*^{H78} mutant, *hs-flp*; *Gmd*^{H78} *FRT42D O-fut1*^{4R6}/*CyO*, *Kr-GFP* males were crossed to *Gmd*^{H78} *FRT42D P{πM}45F*/*CyO*, *Kr-GFP* females.

Constructs

For the O-fut1-ER^{-} construct, three tandem oligonucleotides encoding GSEEQKLISEEDLL were inserted into an artificially created *Bam*HI site before the stop codon of O-fut1 cDNA. *UAS-O-fut1*, *UAS-O-fut1-G3*, *UAS-O-fut1-ER*$^{-}$ and *UAS-ER-CFP* (unpublished, provided by A. Sato, Purde University, West Lafayette, IN) were made by inserting cDNAs encoding wild-type O-fut1, Nti-G3, a mutant O-fut1 lacking its *O*-fucosyltransferase activity (Sasamura et al., 2003), O-fut1-ER^{-}, a mutant O-fut1 lacking a functional ER-retention signal, and ECFP-ER (Clontech), an ECFP with an ER-retention signal, respectively, into pUAST.

Cell culture

S2 cells were cultured, transfected and stained as described previously (Fehon et al., 1990; Sasamura et al., 2003). To express O-fut1-ER^{-} in S2 cells, a cDNA encoding O-fut1-ER^{-} was cloned into pRmHa-3 and introduced into S2 cells. We also used pRmHa-3-Notch (Fehon et al., 1990), pRmHa-3-NotchΔEC (Rebay et al., 1991), pRmHa-3-O-fut1-myc and pRmHa-3-O-fut1-IR (Sasamura et al., 2003). To detect O-fut1 incorporated into Notch-expressing S2 cells, conditioned medium containing O-fut1-ER^{-} was collected from S2 cells transfected with pRmHa-3-O-fut1-ER^{-} and added to S2 cells transfected with pRmHa-3-Notch. After a 20-minute incubation, the cells were fixed and stained as described previously (Fehon et al., 1990). To detect the effect of the O-fut1 knockdown on Notch endocytosis, the transfected S2 cells were incubated in *Drosophila* M3 medium (Sigma) on glass slides coated with concanavalin A (Sigma) for 2 hours at 25°C, anti-Notch extracellular antibody (rat1, 1/500) was added, and the slides were incubated at 4°C for 1 hour. After being washed in M3 medium three times at 4°C, the cells were incubated in M3 medium for 15 minutes at 25°C. In some cases, conditioned medium containing O-fut1-ER^{-} was used in place of the M3 medium, beginning 20 minutes before the antibody was added and continuing until the end of the culture period. The cells were subsequently fixed and stained.

Immunohistochemistry

The primary antibodies used in this study were mouse anti-Notch C17.9C6 (1/500) (Fehon et al., 1990), rat anti-Notch rat1 (1/500, a gift from S. Artavanis-Tsakonas), rat anti-*D*E-cadherin DCAD2 (1/20), guinea pig anti-Hrs (1/1,000) (Lloyd et al., 2002), mouse anti-GAL4 RK5C1 (1/500, Santa Cruz), mouse anti-engrailed (1/1000) (Patel et al., 1989), mouse anti-Wg 4D4 (1/500) (Brook and Cohen, 1996), rat anti-GFP GF090R (1/1000, Nacalai) and mouse anti-Myc MC045 (1/500, Nacalai). An O-fut1 guinea pig antibody was raised against O-fut1 (amino acids 27 to 402) that had six histidines added to the C-terminus and was expressed in SF9 insect cells (used in 1/1000 dilution). Immunostaining of wing discs and Garland cells was performed as previously described (Matsuno et al., 2002). To detect cell-surface Notch, dissected wing discs were incubated in 1/100-diluted rat1 in M3 medium at 4°C for 2 hours. They were rinsed four times with M3 medium at 4°C, and then incubated for 20 minutes or 10 hours in M3 medium. At this point the M3 medium was supplemented with 1 μl/ml 20-OH ecdysone (Sigma) and 1% fetal calf serum (Gibco). The endocytic tracer uptake assay was performed as described (Entchev et al., 2000). Confocal images were taken with LSM5 PASCAL and LSM510 META. We used Auto Deblur (AutoQuant) as the deconvolution tool.

Measurement of cytosolic GDP-L-fucose concentration

GDP-L-fucose levels contained in whole-larva homogenates were measured using previously described procedures with minor modifications (Noda et al., 2002). Briefly, larvae were homogenized in a Dounce homogenizer under crushed ice in 250 μl of 0.25 mol/l sucrose buffer containing Protease Inhibitor Mix/DMSO diluted 1/1000 (Wako, Osaka, Japan), 5 mmol/l adenosine-5-monophosphate (AMP) (pH 7.4) (Wako, Osaka, Japan), 10 mmol/l Tris-HCl (pH 7.4), 10 mmol/l KCl and 10 mmol/l $MgCl_2$. Larva homogenates were spun and the supernatants were subjected to ultracentrifugation at 105,000×***g*** for 1 hour at 4°C to obtain the cytosolic fraction. The protein concentration in these fractions was quantified using a BCA kit (Pierce, IL, USA). In a typical experiment, 120 μg protein from the cytosolic fraction was adjusted to a volume of 20 μl with chilled autoclaved water and then boiled at 100°C for 20 seconds. Then, 8.5 μl ice-cold 200 mmol/l MES-NaOH (pH 7.0) was added, and the samples were spun, mixed with 1 μl 10% Triton X-100 and 0.5 μl (36.8 pmol) GnGn-bi-Asn-4-(2-pyridylamino) butylamine (PABA) (Sigma), subjected to a series of enzymatic digestions and coupled with PABA through a peptide bond and 5 μl purified α1-6 FucT (1050 nmol/l). The mixtures were incubated at 37°C for 2 hours and the reaction was terminated by boiling at 100°C for 1 minute. The samples were then spun at 15,000×***g*** for 10 minutes, and 10 μl of the 35 μl of supernatant was subjected to high performance liquid chromatography for the GDP-L-fucose assay as described (Noda et al., 2003).

Immunoprecipitation and western blotting

Whole-cell extract was prepared from S2 cells as previously described (Sasamura et al., 2003). Anti-Notch (C17.9C6) or anti-Myc (9E10) antibodies were added to cell lysates and immunoprecipitated with Protein G Sepharose 4 Fast Flow (Amersham). The beads were washed five times with an extraction buffer and subjected to western blotting as described (Sasamura et al., 2003). The primary antibodies used for blotting were anti-Notch (C17.9C6) and anti-Myc (9E10).

RESULTS

O-fut1 is required for the endocytic transportation of Notch to the early endosome

Notch is distributed in a honeycomb pattern, which corresponds to the location of the adherens junctions, as judged by the localization of *D*E-cadherin, in the apical region of the wild-type wing disc epithelium (Fig. 1A, upper part; Fig. 1E) (Fehon et al., 1991; Oda et

al., 1994). However, in *O-fut1* homozygous mutant (*O-fut1*$^{-}$) cells, this localization was disrupted, and Notch accumulated in intracellular vesicles, which were distributed mostly in the apical cytoplasm, as reported previously (Fig. 1A-E) (Okajima et al., 2005). This Notch accumulation was cell autonomous in *O-fut1*$^{-}$ cells (Fig. 1B,C). It is proposed that this accumulation is caused by the quality control mechanism, which retains mis-folded Notch in the endoplasmic reticulum (ER) (Okajima et al., 2005). However, we previously showed that cell-surface Notch does not decrease significantly when O-fut1 is knocked down, in the *Drosophila* S2 cell line (Sasamura et al., 2003). Therefore, we decided to determine whether Notch was transported normally to the plasma membrane in *O-fut1*$^{-}$ cells in vivo.

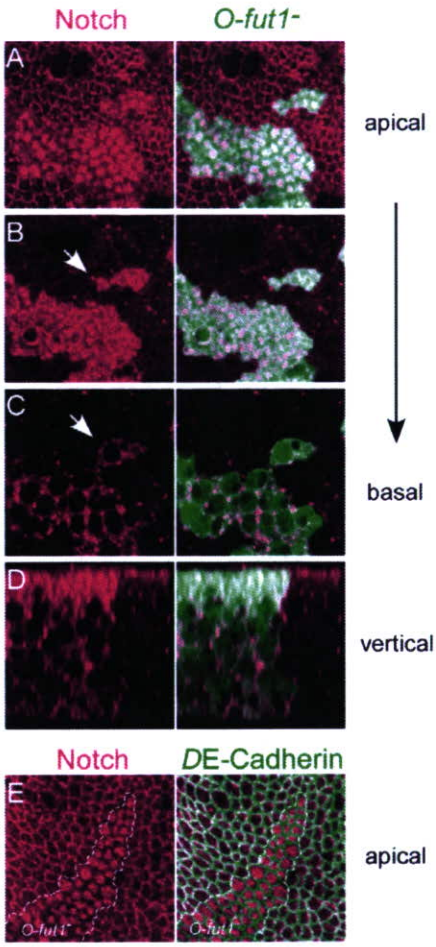

Fig. 1. Notch accumulated in *O-fut1*$^{-}$ cells. (**A-D**) *O-fut1* mutant clones, indicated by the expression of GFP (green), generated by the MARCM method in late third-instar wing discs, and stained with an anti-Notch antibody (C17.9C6, magenta). Apical (A), subapical (B, 1.2 μm beneath the apical level), basal (C, 6.6 μm) and vertical (D) sections of wing disc epithelium are shown. Strong Notch staining was detected in all *O-fut1*$^{-}$ cells. Arrows indicate a small clone surrounded by wild-type cells (B,C). (**E**) Notch failed to localize to the adherens junctions in *O-fut1*$^{-}$ clones. Late third-instar wing discs that contained *O-fut1*$^{-}$ somatic clones were stained with anti-*DE*-Cadherin (green), and anti-Notch antibodies (C17.9C6, magenta). The clone boundary is indicated by a white dashed line.

To detect the fates of the Notch receptors present at the cell surface, we incubated live wing discs from third-instar larvae in medium containing an antibody against the extracellular domain of Notch (rat1) (Le Borgne and Schweisguth, 2003). The antibody was detected in intracellular vesicles in wild-type cells after a 20 minute incubation (Fig. 2C-F, left part of each panel). However, the antibody was not incorporated into these vesicles in live Notch mutant cells, indicating that the antibody specifically labeled Notch under these conditions (Fig. 2A,B). In live *O-fut1*$^{-}$ cells under the same conditions, Notch was detected in intracellular vesicles that were mostly located in the apical region, although these Notch-containing vesicles were smaller and the staining was fuzzy compared with the Notch-containing vesicles in wild-type cells (Fig. 2C-F, at right). These results suggest that Notch was delivered to the plasma membrane in these cells.

We next studied the intracellular vesicles in wild-type and *O-fut1*$^{-}$ cells in more detail. In wild-type cells, most of the Notch-containing endocytic vesicles, visualized by live Notch staining, were labeled by the early endosome marker Hrs (Lloyd et al., 2002) (Fig. 2G-J, right part). In the *O-fut1*$^{-}$ cells, however, live-labeled Notch was not found in the Hrs-positive early endosomes (Fig. 2G-J, left). Furthermore, the live antibody staining for Notch in the intracellular vesicles of the *O-fut1*$^{-}$ cells was much stronger than in the wild-type cells after 10 hours, indicating that Notch was indeed incorporated into the cells by endocytosis but failed to be degraded (Fig. 2K). These results suggested that surface Notch was not transported to the early endosome in *O-fut1*$^{-}$ cells, thereby preventing the trafficking of Notch to the lysosomes, where Notch is degraded (Lu and Bilder, 2005).

Early and late endosomes in the wing disc epithelium can be visualized by fluorescent dextran added extracellularly to the live wing discs (Entchev et al., 2000). Using this system to follow the localization of Notch, we found that the dextran-positive vesicles also stained for Notch (95% of vesicles, n=77) in permeabilized wild-type cells (Fig. 2L, white arrowheads and upper left inset) (Hori et al., 2004). By contrast, in the *O-fut1*$^{-}$ cells, only 6% of the dextran-positive vesicles were also positive for Notch (n=47) (Fig. 2L, open arrowheads and lower right inset). Therefore, Notch failed to be transported to these endosomal compartments. Furthermore, the numbers of dextran-positive endocytic vesicles were equivalent in the wild-type and *O-fut1*$^{-}$ cells, indicating that the *O-fut1* mutation did not affect their formation (Fig. 2L). Together, these observations suggest that O-fut1 is required for the transportation of Notch from early endocytic vesicles to the early endosome. However, we could not identify the early endocytic vesicles in which Notch accumulated in the *O-fut1*$^{-}$ cells. None of the available markers for various endocytic compartments, such as Hook (early endosomes) (Chang et al., 2002), Rab11 (recycling endosomes) (Ullrich et al., 1996; Dollar et al., 2002), rab7-GFP (late endosomes) (Entchev et al., 2000) or ubiquitinylated proteins (aggresomes) (Kopito, 2000) showed co-labeling with Notch in the *O-fut1*$^{-}$ cells (data not shown).

Most Notch was not co-localized with established ER markers in *O-fut1*$^{-}$ cells

Next, we examined whether Notch co-localized with marker proteins for the ER. Protein disulfide isomerase (PDI) is generally involved in protein folding in the ER lumen and is important for quality control (Ferrari and Söling, 1999). A protein trap line, 74-1, expresses PDI-GFP under the control of the original *pdi* promoter and thus accurately reflects the spatiotemporal expression and localization of the PDI protein (Bobinnec et al., 2003). Under a