The WNT receptor Frizzled-8 in pulmonary remodelling and inflammation
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No major role for the Frizzled-8 receptor in allergen-induced airway inflammation and remodelling in vivo

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Chapter 6

Abstract

Objective

We previously showed that the WNT receptor Frizzled-8 (FZD8) is associated with chronic bronchitis and regulates transforming growth factor (TGF)-β-induced remodelling in vitro and in vivo and that the expression of its ligand WNT-5A is increased in lung tissue of asthmatic patients. The objective of this study was to investigate the role of FZD8 in vivo in a mouse model of allergen-induced airway inflammation and remodelling using FZD8 deficient mice.

Methods

Wild-type (WT) and FZD8 knock-out mice (FZD8−/−) were challenged with ovalbumin. mRNA expression and cytokine protein levels were measured in lung tissue homogenates. The number of eosinophils and goblet cells, collagen deposition and the presence of smooth muscle (sm)-α-actin around the airways and vessels were determined by immunohistochemistry on paraffin-embedded lung tissue sections.

Results

Basal mRNA expression of IL-5 and IL-13 was increased, whereas basal IL-4 protein levels and basal collagen deposition were reduced in FZD8−/− mice, indicating a role for FZD8 in developmental mechanisms in the airways. Ovalbumin induced a significant increase in the number of eosinophils and goblet cells, the mRNA expression of MUC5AC, IL-5 and IL-13 as well as IL-5 and IL-13 protein levels in WT mice. Only the ovalbumin-induced mRNA expression of MUC5AC, IL-5 and IL-13 were lower in FZD8−/− mice than in WT mice. This illustrates a different role for FZD8 in developmental versus allergen-induced mechanisms. Ovalbumin challenge induced sm-α-actin around the airways in WT mice, but because of a large variation in the induction of sm-α-actin, we could not study a possible role for FZD8 herein.

Conclusion

This study shows that FZD8 has no major role in the regulation of allergen-induced airway inflammation and that FZD8 is in part required for basal collagen deposition and basal IL-4 protein production in the airways.
Introduction

Asthma is an obstructive airway disease, caused by a combination of environmental and (epi)genetic factors and characterized by chronic airway inflammation, bronchial hyperresponsiveness and airway remodelling [1-3]. Airway inflammation in asthma is mainly eosinophilic in nature and regulated by CD4+ lymphocytes and the T helper (Th2)-type cytokines, interleukin (IL)-4, IL-5 and IL-13 [4-7]. The remodelling process includes structural changes in the lung such as airway smooth muscle hypertrophy and hyperplasia, goblet cell metaplasia, bronchial vascular remodelling as well as basement membrane thickening and subepithelial fibrosis [8-10]. Structural changes in the airways are associated with more severe airway hyperresponsiveness [11], lung function decline and airflow obstruction [12]. The airflow obstruction seen in most asthmatic patients is spontaneously reversible or by treatment with bronchodilators or inhaled corticosteroids. Nevertheless, some patients remain symptomatic despite treatment and the inflammation and remodelling persist in this group of patients [13, 14].

The wingless/integrase-1 (WNT) signalling pathway is a developmental pathway contributing to normal lung development that is re-activated upon repair in adult tissue [15, 16]. WNT ligands are highly conserved between different species. There are 19 mammalian WNT ligands, 10 Frizzled (FZD) receptors and several non-FZD WNT receptors known. WNT ligands bind to their receptors and can either activate the canonical WNT pathway signalling via β-catenin, or signal to one of the noncanonical pathways, for instance to calcium (WNT/Ca²⁺ pathway) or to RhoA/c-Jun N-terminal kinase (JNK) (WNT/PCP (planar cell polarity) pathway) [17-19]. Recent studies indicate the involvement of the WNT signalling pathway in inflammation, tissue repair and remodelling in many organ systems, including the lungs [20-23].

We previously demonstrated that a noncanonical ligand of the WNT signalling pathway, WNT-5A, is increased in lung tissue of asthmatic patients [21]. WNT-5A and its structurally related member WNT-5B were reported to affect inflammation and remodelling via the FZD8 receptor [21, chapter 2]. An important player in remodelling in asthma is transforming growth factor (TGF)-β and TGF-β is known to interact with the WNT signalling pathway [21, 24, chapter 2]. Both WNT-5A, WNT-5B and FZD8 are induced by TGF-β in human airway smooth muscle cells [21] and in human lung fibroblasts [chapter 2] and we showed that FZD8 regulates TGF-β-induced pro-fibrotic signalling [chapter 2]. Furthermore, we showed that FZD8 contributes to the IL-1β- and epidermal growth factor (EGF)-induced inflammatory response in human lung fibroblasts [chapter 4]. IL-1β and EGF contribute to inflammatory processes that underlie asthma pathology [25], therefore these findings suggest that FZD8 may have a role in inflammation and remodelling processes that are seen in asthma. Here, we hypothesize that FZD8 plays a role in the development of allergic asthma. The objective of this study was therefore to investigate the role of FZD8 in a mouse model of allergen-induced airway inflammation and remodelling using FZD8 deficient mice. Our data indicate that FZD8 has a role in developmental mechanisms in the airways, by regulating basal collagen deposition and IL-4 protein secretion in the lungs but that there is no major role for FZD8 in allergen-induced inflammation.
Materials and Methods

Animal studies
All experiments were performed in accordance with the national guidelines and approved by the University of Groningen Committee for Animal Experimentation (approval number 5912E). Heterozygous, inbred, specific-pathogen-free breeding colonies of FZD8+/– mice (C57BL/6;129P2-FZD8tm1Dgen/J), showing no obvious phenotype, were obtained from The Jackson Laboratory (USA). After breeding, homozygous FZD8–/– mice and their wild-type+/+ (WT) littermate controls were used for experiments. Animals were housed under a 12 hour light-dark cycle and received food and water ad libitum. Female FZD8–/– and WT mice were sensitized to ovalbumin via an intraperitoneal injection of 10 µg ovalbumin (Sigma-Aldrich, Zwijndrecht, the Netherlands) emulsified in 1.5 mg aluminum hydroxide (Aluminject®, Pierce Chemical, Etten-leur, the Netherlands) and diluted to 200 µl with saline. Sensitization occurred on day 1, 14 and 21. Subsequently, mice were challenged with either saline (control animals) or 1% ovalbumin aerosols in saline. Mice were challenged by whole body exposure for twenty minutes twice a week on consecutive days for four weeks in a Perspex exposure chamber (9 liters) by a De Vilbiss nebulizer (type 646; De Vilbiss, Somerset, PA) driven by an airflow of 40 L/min providing aerosols with an output of 0.33 ml/min, as described previously [26, 27]. Based on previous studies using this model [28] in our institute [26, 27], ten mice were included in each group.

Tissue collection
Twenty-four hours after the last ovalbumin challenge, mice were euthanized by subcutaneous injection with a mixture of medetomidine (0.5 mg/kg Dormitor®, Orion Pharma, Mechelen, Belgium) and ketamine (40.0 mg/kg, Alfasan, Woerden, the Netherlands) followed by exsanguination. Lung tissue was snap frozen and stored at -80°C for further analysis of cytokine protein levels (left lung), mRNA expression (post caval lobe) and immunohistochemistry (middle and inferior lobe). The superior lobe was fixed in formalin and embedded in paraffin for immunohistochemistry. Preceding further analysis, lungs were mechanically crushed under liquid nitrogen and suspended in mRNA lysis buffer for mRNA analysis or suspended in Tris-lysis buffer (pH 7.4) with protease inhibitors (cOmplete, Mini EDTA-free, Roche Life Sciences, Indiana, IN, USA) and subsequently sonicated for cytokine analysis. Samples were stored at -80°C until further use.

mRNA analysis
Total mRNA was isolated from lung tissue homogenates using the NucleoSpin® RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The eluted mRNA was quantified using spectrophotometry (Nanodrop, Thermo Scientific™, Wilmington, DE, USA). Equal amounts of mRNA (1 µg) were reverse transcribed using the Reverse Transcription System (Promega Benelux b.v., Leiden, the Netherlands). cDNA was stored at -20°C until further use. mRNA expression was determined using real-time PCR, performed with the Illumina Eco Personal qPCR System (Westburg, Leusden, the Netherlands). The cycle parameters were denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds for 40 cycles. Primer sets are listed in table 1. Real-time PCR data were analyzed using the comparative cycle threshold (Ct) method. Ct is the amplification cycle number. The amount of target gene was normalized against the endogenous
housekeeping gene 18S ribosomal RNA (designated as ΔCt). Experimental conditions were normalized against the average of ΔCt values in the WT group treated with saline (designated as ΔΔCt). Relative differences were determined using the equation $2^{-(ΔΔCt)}$.

### Table 1 - Mouse primers used to determine specific genes of interest.

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>FZD1</td>
<td>Forward 5’ CAA GGT TTA CGG GCT CAT GT 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ GTA ACA GCC GGA CAG GAA AA 3’</td>
</tr>
<tr>
<td>FZD2</td>
<td>Forward 5’ CCG TCT CTG CAT CCT CAC AT 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ TAG CAG CCG GAC AGA AAG AT 3’</td>
</tr>
<tr>
<td>FZD3</td>
<td>Forward 5’ GAA GCA AAG CAG GGA GTG TC 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ ATG CTG CCG TGA GGT AGT CT 3’</td>
</tr>
<tr>
<td>FZD4</td>
<td>Forward 5’ CTG CAG CAT GCC TAA TGA GA 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ CGT CTG CCT AGA TGC AAT CA 3’</td>
</tr>
<tr>
<td>FZD5</td>
<td>Forward 5’ AGC CAA ATG AGG CAC ATA CC 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ TCT CCT TTT GCC AGC GTT AT 3’</td>
</tr>
<tr>
<td>FZD6</td>
<td>Forward 5’ TCC GAC GCT TGA AGA AAA CT 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ CAA CCC CAG GTC CTC AGA TA 3’</td>
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<tr>
<td>FZD7</td>
<td>Forward 5’ ATC ATC TTC CTG TCG GGT TG 3’</td>
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<tr>
<td></td>
<td>Reverse 5’ AAG CAC CAT GAA GAG GAT GG 3’</td>
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<tr>
<td>FZD8</td>
<td>Forward 5’ TCC GTT CAG TCA TCA AGC AG 3’</td>
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<tr>
<td></td>
<td>Reverse 5’ CGG TTG TGC TGC TCA TAG AA 3’</td>
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<td>FZD9</td>
<td>Forward 5’ CCA GCT GTC AGC AGC GTA CA 3’</td>
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<td></td>
<td>Reverse 5’ CAC TCC CTG CAT GAG ACA GA 3’</td>
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<td>FZD10</td>
<td>Forward 5’ TGG TAC GCA TAG GGG TCT TC 3’</td>
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<td></td>
<td>Reverse 5’ TCA GGC AGT CAG GTG TCT TG 3’</td>
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<td>WNT-5A</td>
<td>Forward 5’ CAA ATA GGC AGC CGG GAC 3’</td>
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<tr>
<td></td>
<td>Reverse 5’ CTC TAG CGT CCA CCA ACT CC 3’</td>
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<tr>
<td>WNT-5B</td>
<td>Forward 5’ GGT TCC ACT GGT GTT GCT TT 3’</td>
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<tr>
<td></td>
<td>Reverse 5’ AGA CTT TTG TGA GGC GGA GA 3’</td>
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<td>IL-4</td>
<td>Forward 5’ TCC TCA CAGCAA CGA AGG ACA 3’</td>
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<td></td>
<td>Reverse 5’ TCG CCA CAC TCT TTT TGG 3’</td>
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<td>IL-13</td>
<td>Forward 5’ TGG CGG GTT CTG TGT AGC 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ CTT GCG GTT ACA GAG GCC AT 3’</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>Forward 5’ GAG ATG GAG GAT CTG GGT CA 3’</td>
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<td></td>
<td>Reverse 5’ GCA GAA GCA GGG AGT GTG AG 3’</td>
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<tr>
<td>18S rRNA</td>
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<tr>
<td></td>
<td>Reverse 5’ CCT CCA ATG GAT CCT GCT TA 3’</td>
</tr>
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</table>

**Cytokine analysis**

A Luminex® Screening Assay was performed on the lung tissue homogenates to determine cytokine protein levels. The amount of protein in lung tissue homogenates was determined according to Pierce® BCA protein determination assay following the manufacturer’s instructions. Cytokine protein levels were measured using a Mouse Luminex® Screening Assay measuring IL-4, IL-5 and IL-13 according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA) on a Luminex 100 system using Starstation software (Applied Cytometry Systems, Sheffield, UK).

**Immunohistochemistry**

Transverse cross-sections of 5 μm thick were used for morphometric analyses. Paraffin-embedded sections were stained for cyanide resistant endogenous peroxidase activity
with diaminobenzidine (D5637; Sigma-Aldrich, Zwijndrecht, the Netherlands) to visualize eosinophils. The number of eosinophils in the airway wall was counted and expressed as number of cells per mm basement membrane. Paraffin-embedded sections were stained for goblet cells with Periodic Acid Schiff’s (PAS; 395B; Sigma-Aldrich, Zwijndrecht, the Netherlands). PAS-positive cells were counted and expressed per mm basement membrane. Paraffin-embedded sections were stained for collagen with Sirius Red (80115, batch 101/WSC; Klinipath, Duiven, the Netherlands). Collagen deposition around the airway was quantified using ImageJ [29] and expressed as mm² per mm² basement membrane. Smooth muscle (sm)-α-actin was stained on paraffin-embedded sections using anti-mouse sm-α-actin rabbit polyclonal antibody (1:200; ab5645; Abcam, Cambridge, UK) and visualized using a horseradish-peroxidase-conjugated secondary antibody goat anti-rabbit (1:300; A0545; Sigma-Aldrich, Zwijndrecht, the Netherlands) and diaminobenzidine. The presence of sm-α-actin around the airways and vessels was quantified using ImageJ [29]. The surface of positively stained tissue was expressed as mm² per mm² basement membrane (airways) or mm² per mm² internal vessel diameter (vessels).

**Statistical analysis**

Statistical analysis was performed with SigmaPlot™ software (Systat Software Inc., San Jose, CA, USA). All real-time PCR data were log-transformed before analysis. For comparison between multiple conditions within the different groups of mice, two-way ANOVA was used, followed by a post hoc analysis using the Student-Newman-Keuls multiple comparisons test. For comparison between two conditions within the different groups of mice, a two-tailed Mann-Whitney test was used. P < 0.05 was considered significant.

**Results**

**WNT and FZD mRNA expression in FZD8⁻/⁻ mice compared to WT mice**

To characterize the expression of all FZD receptors in the FZD8⁻/⁻ mice, we first investigated mRNA expression of FZD1-10. FZD8 mRNA expression was not detectable in FZD8⁻/⁻ mice. We found no significant differences between the basal mRNA expression of FZD receptors in FZD8⁻/⁻ mice compared to WT mice, we observed that basal FZD5 mRNA expression tends to be reduced (figure 1A). Ovalbumin did not cause a significant change in the expression of FZD1-10 in WT mice, although the mRNA expression of FZD1, FZD2 and FZD7 tended to increase, while the mRNA expression of FZD4 and FZD6 tended to decrease (figure 1A). In FZD8⁻/⁻ mice, ovalbumin challenge induced a reduction in FZD1, FZD2, FZD3, FZD4 and FZD7 mRNA expression, compared to saline treated FZD8⁻/⁻ mice and a reduction in FZD1, FZD2, FZD3, FZD4 and FZD7 mRNA expression compared to ovalbumin-challenged WT mice (figure 1A). Because WNT-5A and WNT-5B are known ligands for FZD8, we also investigated WNT-5A and WNT-5B mRNA expression. We found no significant difference between saline challenged WT mice and saline challenged FZD8⁻/⁻ mice (figure 1B). Ovalbumin challenge did not change WNT-5A and WNT-5B mRNA expression in WT mice and induced a reduction in WNT-5A mRNA expression in FZD8⁻/⁻ mice compared to ovalbumin challenged WT mice (figure 1B).
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Figure 1: Basal and ovalbumin-induced WNT and FZD mRNA expression in WT and FZD8$^{-/-}$ mice. To characterize the FZD8$^{-/-}$ mice, we investigated FZD1-10, WNT-5A and WNT-5B mRNA expression. (A) mRNA expression of FZD receptors relative to the average expression in the saline challenged WT group. FZD8 mRNA expression in FZD8$^{-/-}$ mice was not detectable. (B) mRNA expression of WNT-5A and WNT-5B relative to the average expression in the saline challenged WT group. Data represent mean ± s.e.m. of 8-10 mice per group. # $p < 0.05$ ## $p < 0.01$ ### $p < 0.001$ compared to ovalbumin challenged WT mice, $\$ p < 0.05$ $\$$ $p < 0.01$ compared to saline challenged FZD8$^{-/-}$ mice (two-way ANOVA with Student-Newman-Keuls multiple comparisons post hoc test).
Airway inflammation
To investigate whether FZD8 regulates allergen-induced airway inflammation, we investigated the number of eosinophils in the airway wall and the expression of Th2-type cytokines in lung tissue. There were a low number of eosinophils present in saline challenged mice in both WT and FZD8\(-/-\) mice, without significant difference (figures 2A-B; table 2). IL-4 mRNA expression was not significantly different between the groups, while IL-5 and IL-13 mRNA expression was increased in FZD8\(-/-\) mice compared to WT mice (figures 2C-E; table 2). Ovalbumin challenge induced a significant increase in the number of eosinophils in both WT mice and FZD8\(-/-\) mice (figures 2A-B), without significant differences between the groups. Furthermore, ovalbumin challenge tended to increase IL-4 mRNA expression and induced a significant increase in the mRNA expression of IL-5 and IL-13 in WT mice. The latter effect was significantly less in FZD8\(-/-\) mice, but ovalbumin challenge still caused a significant increase (IL-5: p = 0.016 for the interaction of ovalbumin challenge and genotype; IL-13: p = 0.01 for the interaction of ovalbumin challenge and genotype; figures 2C-E; table 2). At the protein level, IL-4 in lung tissue homogenates was reduced in saline treated FZD8\(-/-\) mice compared to saline treated WT mice, while IL-5 and IL-13 protein levels were not detectable (figures 2F-H; table 2). Ovalbumin challenge increased IL-5 and IL-13 but not IL-4 protein levels in lung tissue homogenates in WT mice and absolute levels were not different from FZD8\(-/-\) mice (figures 2F-H; table 2). The relative induction of IL-4 protein levels after ovalbumin challenge was higher in FZD8\(-/-\) mice, with only a significant increase in IL-4 protein levels upon ovalbumin challenge in FZD8\(-/-\) mice (p = 0.038 for the interaction of ovalbumin challenge and genotype; figure 2F; table 2).

### Table 2 - Ovalbumin-induced airway inflammation in WT and FZD8\(-/-\) mice.

<table>
<thead>
<tr>
<th></th>
<th>WT Saline</th>
<th>Ovalbumin</th>
<th>FZD8(-/-) Saline</th>
<th>Ovalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosinophils</td>
<td>1.00 ± 0.21</td>
<td>13.7 ± 4.03 **</td>
<td>4.22 ± 2.27</td>
<td>18.5 ± 2.73 $$$</td>
</tr>
<tr>
<td>IL-4 mRNA</td>
<td>2.34 ± 0.89</td>
<td>5.44 ± 1.06</td>
<td>2.80 ± 0.91</td>
<td>3.12 ± 1.59</td>
</tr>
<tr>
<td>IL-5 mRNA</td>
<td>1.45 ± 0.28</td>
<td>16.44 ± 4.00 ***</td>
<td>3.60 ± 1.08 *</td>
<td>10.48 ± 3.04</td>
</tr>
<tr>
<td>IL-13 mRNA</td>
<td>1.37 ± 0.42</td>
<td>15.03 ± 3.19 ***</td>
<td>2.90 ± 0.66 *</td>
<td>9.69 ± 3.05 $</td>
</tr>
<tr>
<td>IL-4 protein</td>
<td>0.027 ± 0.003</td>
<td>0.032 ± 0.003</td>
<td>0.015 ± 0.002 **</td>
<td>0.031 ± 0.003 $$$</td>
</tr>
<tr>
<td>IL-5 protein</td>
<td>n.d.</td>
<td>0.009 ± 0.004 **</td>
<td>n.d.</td>
<td>0.011 ± 0.003 $</td>
</tr>
<tr>
<td>IL-13 protein</td>
<td>n.d.</td>
<td>0.0008 ± 0.0005</td>
<td>n.d.</td>
<td>0.003 ± 0.0018</td>
</tr>
</tbody>
</table>

Eosinophils and mRNA represents fold increase compared to WT mice challenged with saline; protein represents pg/ml. * p < 0.05 ** p < 0.01 *** p < 0.001 compared to saline challenged WT mice, $ p < 0.05 $$ p < 0.01 $$$ p < 0.001 compared to saline challenged FZD8\(-/-\) mice (two-way ANOVA with Student-Newman-Keuls multiple comparisons post hoc test); n.d. = not detectable.

**Figure 2: Airway inflammation in WT and FZD8\(-/-\) mice.** To investigate whether FZD8 regulates airway inflammation, we investigated the number of eosinophils in the airway wall and the mRNA expression and protein secretion of Th2-type cytokines in lung tissue. (A) The number of eosinophils around the airways was counted and expressed as number of cells per mm basement membrane. (B) Representative staining for eosinophils in the airway wall in WT and FZD8\(-/-\) mice. (C) mRNA expression of IL-4 relative to the average expression in the saline challenged WT group. (D) mRNA expression of IL-5 relative to the average expression in the saline challenged WT group; p = 0.016 for the interaction of ovalbumin challenge and genotype. (E) mRNA expression of IL-13 relative to the average expression in the saline challenged WT group; p = 0.01 for the interaction of ovalbumin challenge and genotype. (F) Protein secretion of IL-4 (pg/ml); p = 0.038 for the
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Figure 2

A interaction of ovalbumin challenge and genotype. (G) Protein secretion of IL-5 (pg/ml). (H) Protein secretion of IL-13 (pg/ml). Data represent mean ± s.e.m. of 8-10 mice per group. * p < 0.05 ** p < 0.01 *** p < 0.001 compared to saline challenged WT mice, $ p < 0.05 $$$ p < 0.01 $$$ $ p < 0.001 compared to saline challenged FZD8−/− mice (two-way ANOVA with Student-Newman-Keuls multiple comparisons post hoc test).
**Goblet cell metaplasia**

To investigate the role of FZD8 in goblet cell metaplasia, lung tissue sections were stained with a PAS staining and MUC5AC mRNA expression was determined in lung tissue homogenates. There were no goblet cells detectable in saline challenged mice in both WT and FZD8⁻/⁻ mice, while MUC5AC mRNA expression was detectable, but not significantly different between the genotypes (figures 3A-C). Ovalbumin induced an increase in MUC5AC mRNA that was significantly less in FZD8⁻/⁻ mice compared to WT mice, but ovalbumin challenge still caused a significant increase in FZD8⁻/⁻ mice (27.61 ± 8.63 fold compared to 47.07 ± 7.17 fold respectively, \( p = 0.0736 \) for the interaction of ovalbumin challenge and genotype; figure 3C). However, there were no differences in ovalbumin-induced goblet cells as indicated by PAS staining in FZD8⁻/⁻ and WT mice: (0.022 ± 0.006 and 0.0025 ± 0.006 goblet cells per mm basement membrane respectively; figures 3A-B). This indicates that FZD8 regulates ovalbumin-induced MUC5AC mRNA transcription, but not ovalbumin-induced goblet cell metaplasia.

**Figure 3: Goblet cell metaplasia in WT and FZD8⁻/⁻ mice.** To investigate the effect of FZD8 on goblet cell metaplasia, lung tissue sections were stained with Schiff’s staining and MUC5AC mRNA expression was determined in lung tissue homogenates. (A) PAS-positive cells were counted and expressed per mm basement membrane. (B) Representative staining for goblet cells in the airways of WT and FZD8⁻/⁻ mice. (C) MUC5AC mRNA expression relative to the average expression in the saline challenged WT group; \( p = 0.0736 \) for the interaction of ovalbumin challenge and genotype. Data represent mean ± s.e.m. of 8-10 mice per group. *** \( p < 0.001 \) compared to saline challenged WT mice, $$$ \( p < 0.001 \) compared to ovalbumin challenged WT mice, $\# \ p < 0.05$ compared to saline challenged FZD8⁻/⁻ mice (two-way ANOVA with Student-Newman-Keuls multiple comparisons post hoc test).
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**Figure 4: Airway remodelling in WT and FZD8<sup>−/−</sup> mice.**

To determine the effect of FZD8 on remodelling of the airway smooth muscle around the airways and vessels, sections were stained for collagen and sm-α-actin. (A) Collagen deposition was expressed as mm<sup>2</sup> per mm<sup>2</sup> basement membrane; p = 0.094 for the difference between genotypes. p = 0.015 in a Mann-Whitney on collagen deposition in saline challenged FZD8<sup>−/−</sup> mice compared to saline challenged WT mice. (B) Representative staining for collagen deposition around the airways in WT and FZD8<sup>−/−</sup> mice. (C) The surface of positively stained tissue around the airways of sm-α-actin was expressed as mm<sup>2</sup> per mm<sup>2</sup> basement membrane; p = 0.061 for the interaction between ovalbumin challenge and genotype. p = 0.015 in a Mann-Whitney on sm-α-actin around the airways in saline challenged WT mice compared to ovalbumin challenged WT mice. (D) The surface of positively stained tissue around the vessels of sm-α-actin was expressed as mm<sup>2</sup> per mm<sup>2</sup> internal vessel diameter; p = 0.097 for the interaction between ovalbumin challenge and genotype. (E) Representative staining for sm-α-actin around the airways and vessels in WT and FZD8<sup>−/−</sup> mice. Data represent mean ± s.e.m. of 8-10 mice per group.
Airway and vascular wall thickening
To determine the role of FZD8 in remodelling around the airways and vessels, lung tissue sections were stained for collagen and sm-α-actin. Collagen deposition was reduced in saline challenged FZD8-/- mice compared to saline challenged WT mice (p = 0.015 on collagen deposition in saline challenged FZD8-/- mice compared to saline challenged WT mice). Ovalbumin did not cause an increase in collagen deposition in either genotype (figures 4A-B). The expression of sm-α-actin around the airways and the vessels was not significantly different between FZD8-/- mice and WT mice in saline challenged animals. Ovalbumin increased sm-α-actin around the airways (p = 0.015 on saline challenged WT mice compared to ovalbumin challenged WT mice; figures 4C,E) and tended to induce an increase in smooth muscle thickening around the vessels (figures 4D-E). However, overall statistics showed no statistical differences between the different genotypes or the interaction between genotype and ovalbumin challenge on either airway or vascular smooth muscle content.

Discussion
Our data suggest that FZD8 has no major role in allergen-induced inflammation and a limited role in developmental mechanisms in the airways. We demonstrate that FZD8-/- mice do not display differences in basal eosinophil counts, mucus producing cells and sm-α-actin around the airways and vessels. We found that basal IL-4 protein levels as well as basal collagen deposition are reduced in FZD8-/- mice. On the other hand, FZD8-/- mice do not display differences in ovalbumin-induced eosinophil counts, mucus producing cells. Ovalbumin-induced mRNA expression of IL-5, IL-13 and MUC5AC were reduced in FZD8-/- mice compared to WT mice.

We previously demonstrated that FZD8 has pro-inflammatory properties, regulating IL-1β- and EGF-induced IL-6 and chemokine ligand CXCL8 secretion from human lung fibroblasts, as well as acute cigarette smoke-induced airway inflammation in the same FZD8-/- mice as used in the current study [chapter 4]. Cigarette smoke-induced airway inflammation and neutrophil and lymphocyte cell counts were reduced in FZD8-/- mice [chapter 4]. As IL-1β and EGF contribute to disease mechanisms that underlie asthma pathology [25], we were interested to see if FZD8 is also involved in allergen-induced airway inflammation. However, the current study did not reveal a full-blown role for FZD8 in allergen-induced inflammation as allergen-induced eosinophil counts and mucus producing cells were not reduced in FZD8-/- mice compared to WT mice and the reduction on inflammatory cytokines and MUC5AC mRNA expression was only minor and not translated in reduced goblet cell numbers.

The differences that we found between the role of FZD8 in these different models can obviously be explained by the fact that ovalbumin induces eosinophilic inflammation which is mainly regulated via the Th2-type cytokine IL-5, whereas cigarette smoke-induced neutrophilic inflammation is established mainly via CXCL8 that is produced by structural as well as immune cells. Although mRNA levels of IL-5 were reduced in the ovalbumin-challenged FZD8-/- mice, this did not result in detectable reduction of IL-5 protein levels nor a reduced number of eosinophils.
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IL-13 is an important player in mucus regulation in multiple airway diseases [30]. We found that FZD8 is not unambiguously involved in basal and ovalbumin-induced IL-13 mRNA expression. Its absence caused an increase in basal IL-13 mRNA expression and a decrease in ovalbumin-induced IL-13 mRNA expression, however this is not translated to a change in IL-13 protein expression. In addition, we found that ovalbumin-induced MUC5AC mRNA expression is less in FZD8−/− mice compared to WT mice. However, this effect was not represented by a change in the number of goblet cells after ovalbumin challenge between WT and FZD8−/− mice. These findings on IL-13 mRNA and protein expression, MUC5AC mRNA expression and the number of goblet cells suggests that FZD8 is specifically involved in the induction of mucin mRNA expression but not via IL-13 and not in the actual differentiation or turn-over of goblet cells. We previously showed that FZD8 is involved in IL-1β- and EGF-induced IL-6 and CXCL8 secretion by fibroblasts and that these cytokines induce MUC5AC mRNA expression in differentiated airway epithelial cells [chapter 4]. For CXCL8 but not for IL-6, this increase in MUC5AC mRNA expression was accompanied by an increase in goblet cell number [chapter 4], indicating that via this signalling route FZD8 can regulate the number of goblet cells.

Our data imply that TGF-β and its mediators, including FZD8 signalling, play distinct roles in basal inflammation and repair mechanisms than in allergen-induced mechanisms. While we did not find a clear role for FZD8 in ovalbumin-induced Th-2-like-cytokine protein levels in lung tissue homogenates, we found that specifically basal IL-4 protein levels are significantly reduced in FZD8−/− mice compared to WT mice. One explanation that FZD8 has a role in IL-4 protein expression and not in IL-5 or IL-13 protein expression could be that in addition to infiltrated lymphocytes, IL-4 can be secreted by human lung fibroblasts [31-34]. This is not the case for IL-5 and IL-13. FZD8 is mainly expressed on mesenchymal cells in the airways [21, 35] and FZD8 knockdown attenuated basal IL-4 secretion from MRC-5 human lung fibroblasts in vitro (data not shown). Therefore, we speculate that the specific reduction in IL-4 levels at baseline is the consequence of the downregulation of FZD8 on fibroblasts, since only IL-4 but not IL-5 and IL-13 can be secreted by fibroblasts. In addition, the fact that we found an increase in basal IL-5 and IL-13 mRNA expression in FZD8−/− mice, while ovalbumin-induced IL-5 and IL-13 mRNA expression were reduced in FZD8−/− mice also shows that FZD8 signalling differentially regulates basal inflammatory mechanisms and allergen-induced mechanisms. Of interest, TGF-β/Smad3 signalling has also been shown to have differential effects on basal and allergen-induced cytokine production in vivo [36, 37], as Anthoni et al. [38] found a basal increase in Th2-type cytokine levels and a reduction in ovalbumin-induced IL-5 and IL-13 but not IL-4 release in Smad3−/− mice, with low levels of IL-13. This is in line with our findings where regulation of FZD8 seems to be different between IL-4, IL-5 and IL-13, the finding that IL-5 and IL-13 protein levels variate between mice and the finding that FZD8 knock-out increased basal mRNA expression of IL-5 and IL-13 while we found a reduction in ovalbumin-induced mRNA expression of these cytokines.

Remodelling is an important contributor to asthma pathology, with TGF-β being a key growth factor. We were interested in the effects of FZD8 in airway remodelling, because we previously found that the expression of one of the ligands for the FZD8 receptor, WNT-5A, is increased in lung tissue of asthmatic patients [21]. In addition, FZD8 regulates TGF-β-induced remodelling in airway smooth muscle cells [21] and lung fibroblasts [chapter 2], as well as TGF-β-induced pro-fibrotic signalling in vivo and in
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vitro [chapter 2]. Using this ovalbumin model, we previously found an increase in the remodelling parameters, such as an increase in collagen deposition and sm-α-actin around the airways and vessels [26, 27]. Here, we found an increase in sm-α-actin in WT mice after ovalbumin challenge, however we did not find a difference for the interaction between ovalbumin challenge and genotype in the overall statistical test. Therefore, we cannot draw conclusions about the role of FZD8 on airway smooth muscle thickening. Interestingly, we found that basal collagen deposition was lower in FZD8–/- mice. This is in line with our findings on the reduced basal IL-4 protein levels in FZD8–/-mice as IL-4 has a pro-fibrotic role and has been linked to collagen production [32, 39]. Thus, while we cannot define a role for FZD8 in allergen-induced remodelling, our data indicate a role for FZD8 in developmental mechanisms of modeling in the airways.

It is not likely that the lack of effect of FZD8 knockout on ovalbumin-induced inflammation could be due to compensation of other factors of the WNT signalling pathway as basal mRNA expression of FZD receptors as well as WNT-5A and WNT-5B was not different in FZD8–/- mice compared to WT mice. However, we could not test this at the protein level due to the absence of suitable antibodies. In addition, we found a differential expression of FZD receptors and WNT-5A mRNA in FZD8–/- mice compared to WT mice upon ovalbumin challenge. We found that FZD1, FZD2 and FZD7 tend to increase after ovalbumin challenge, while FZD4 and FZD6 tend to decrease in WT mice. In the FZD8–/- mice, ovalbumin caused a decrease in mRNA expression of these FZD receptors and also of FZD3 and WNT-5A. These data suggest that FZD8 regulates the expression of these FZD receptors and WNT-5A after ovalbumin challenge. This difference in regulation could be explained by the different clusters within the family of FZD receptors. FZD receptors stem from 4 clusters that show overlap in amino acid structure [40]. FZD5 and FZD8 show 70% overlap, FZD1, FZD2 and FZD7 show 75% overlap, FZD4, FZD9 and FZD10 show 65% overlap and FZD3 and FZD6 show 50% overlap. We propose that FZD8 differentially regulates the gene transcription of these FZD receptor clusters.

Taken together, we show that whereas FZD8 has no major role in the regulation of allergen-induced inflammation in the airways, FZD8 is in part required for developmental mechanisms in the airways with respect to basal collagen deposition and basal IL-4 protein production.

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