Manipulation of Dipeptidylpeptidase 10 in mouse and human in vivo and in vitro models indicates a protective role in asthma.

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ABSTRACT

We previously identified dipeptidyl-peptidase 10 (DPP10) on chromosome 2 as a human asthma susceptibility gene, through positional cloning. Initial association results were confirmed in many subsequent association studies but the functional role of DPP10 in asthma remains unclear. Using the MRC Harwell N-ethyl-N-nitrosourea (ENU) DNA archive, we identified a point mutation in Dpp10 that caused an amino acid change from valine to aspartic acid in the β propeller region of the protein. Mice carrying this point mutation were recovered and a congenic line was established (Dpp10145D). Macroscopic examination and lung histology revealed no significant differences between wild-type and Dpp10145D/145D mice. However, after house dust mite (HDM) treatment, Dpp10 mutant mice showed significantly increased airway resistance in response to 100 mg/ml methacholine. Total serum IgE levels and bronchoalvelar lavage (BAL) eosinophil counts were significantly higher in homozygotes than control mice after HDM treatment. DPP10 protein is present in airway epithelial cells and altered expression is observed in both tissue from asthmatic patients and in mice following HDM challenge. Moreover knockdown of DPP10 in human airway epithelial cells, results in altered cytokine responses. These results show that a Dpp10 point mutation leads to increased airway responsiveness following allergen challenge and provide biological evidence to support previous findings from human genetic studies.

Key words:
DPP10, point mutation, IgE, airway resistance, asthma
INTRODUCTION

Asthma is characterized by intermittent inflammation of the large airways in the lungs with symptoms of wheeze and shortness of breath. The disease is caused by a combination of genetic and environmental factors. Previously, we have established that polymorphisms in \textit{DPP10} on human chromosome 2 were associated with asthma traits through positional cloning (1). These associations have since been replicated in different ethnic populations worldwide (2-6). To date, \textit{DPP10} is the only gene found to show asthma association by both positional cloning and genome-wide association studies (GWAS) (6). \textit{DPP10} encodes a single-pass type II membrane protein that is a member of the S9B family in clan SC of the serine proteases. It has no detectable protease activity in mammals due to the absence of a conserved serine residue normally present in the catalytic domain of these proteases (7). Instead, DPP10 has been shown to bind specific voltage-gated potassium channels altering their expression and biophysical properties (8). Although DPP10 has been associated with asthma in both genome-wide and wet-lab studies (1, 9, 10), its functional role in asthma is almost completely unknown, due in large part to the absence of any genetic models.

Mouse mutants are powerful experimental tools for the study of complex diseases, such as asthma. They have been of great benefit for mapping quantitative traits (11, 12), and serve as experimental tools to dissect the functional roles of genes \textit{in vivo} (13). \textit{Dpp10}^{I45D} mice were generated using N-ethyl-N-nitrosourea (ENU) induced mutagenesis of the mouse genome, which is a conventional method employed for functional analysis (14).

To investigate the function of DPP10 \textit{in vivo}, we obtained full-length mouse \textit{Dpp10} cDNA. We then sequenced four out of the 26 exons of \textit{Dpp10} in 3840 mouse DNA samples from the UK MRC Harwell archive of ENU mutagenized F1 DNA samples. The four exons chosen for sequencing include the regions encoding the trans-membrane and β propeller domains of \textit{Dpp10}, both of which are thought to be required for proper protein function (1). Here, we report the establishment of a novel mouse mutant carrying a DPP10 point mutation and its effects on experimental asthma.
METHODS

**Full-length cDNA of Dpp10 and exon sequencing**

A mouse cDNA clone (BE862767) was obtained and extended 5’ and 3’ ends to a full-length cDNA using RACE (CLONTECH). The primary transcript encodes a novel 2,370 bp open reading frame (ORF) with a predicted peptide sequence of 789 residues. To identify mutations in Dpp10, we screened 4 exons of Dpp10 (2, 5, 6 and 7) in 3840 mutagenised BALB/C DNA samples from the UK MRC Harwell ENU archive by sequencing the PCR products. Exon 2 encodes the trans-membrane amino acids and exon 5, 6, and 7 encode the first and second beta propellers of the peptide. PCR primer sequences are listed in supplemental table 1. PCR conditions were as follows: 35 cycles consisting of (i) 60 s at 94 °C, followed by (ii) 60 s at 50–60 °C and then (iii) 30 s at 72 °C. PCR products from all 3840 DNA samples were purified with Millipore purification plates and sequenced using ABI 3700 sequence machine. Sequence traits were aligned and DNA samples carrying mutations were identified. The mutation predicted to most likely affect function was used to establish the DPP10 mouse line.

**Mouse line recovery and maintenance**

Mice were housed and maintained in accordance with the rules and regulations of the UK Animals (Scientific Procedures) Act 1986, and the Harwell Animal Welfare and Ethical Review Body (AWERB). Mice were maintained in specific pathogen-free conditions and provided food and water ad libitum. Sperm containing the Dpp10 mutation identified above was recovered from the parallel Harwell sperm archive and used in IVF with C3H females to obtain live mice carrying this Dpp10 mutation. Progeny born following IVF were genotyped using a diagnostic restriction enzyme digest followed by PCR as follows: (i) 60 s at 94 °C, (ii) 60 s at 55 °C and (iii) 30 s at 72 °C for 32 cycles. PCR products were digested with the HPYChe4IV enzyme (2u for 3 hours), then run on a 2% agarose gel to determine the size of products. The mutant Dpp10 band was 194 bp and wild-type DNA was cut into 2 fragments of 127
and 62 bp. We subsequently established a congenic Dpp10 line by backcrossing these mice to CH3 for 10 generations.

Histology and immunostaining of lung tissues

Adult human lung tissue was obtained from the Biobank of the Respiratory Biomedical Research Unit (BRU), Royal Brompton and Harefield NHS Foundation Trust. The study was approved by the Trust’s Ethics Committee (ethics reference number 10/H0504/9). Informed written consent was obtained from all study subjects. Control samples were obtained from healthy, non-asthmatic volunteers. Four micrometer paraffin sections or 10 micrometer cryosections were stained with haematoxylin and eosin (H&E), Mauritius scarlet blue (MSB) to identify collagen or immunostained using previously established protocols (15) Lung tissue processing and immunohistochemistry were performed as previously described (16). Antibody dilutions were: aquaporin-5 1:400, CC-10 1:500, Santa Cruz; pro-SP-C 1:1000, Chemicon; alpha smooth muscle actin (SMA) 1:1000, Lab Vision. Incubations were overnight at 4°C. For CC10 immunostaining, antigen retrieval using citrate buffer (PH6) was required to unmask antigens prior to immunodetection. Antibodies were detected with either universal (alpha SMA, pro-SP-C) or goat (CC10, Aquaporin-5) ABC elite staining kit (Vector Labs). Rabbit anti DPP10 was obtained from Santa Cruz Biotechnology (sc-174156) or Aviva Systems Biology (OAAB05596) and used at 1:100 dilution. Controls, where the primary antibody was omitted, were included in each immunostaining experiment.

Quantifying percentage of airway cells with apical Dpp10 immunostaining

The total number of cells in individual airways was determined by counting the number of haemotoxylin stained nuclei. The percentage of cells with apical Dpp10 staining in each airway was determined by expressing the number of cells with brown staining visible at the apical surface of cells (i.e. immediately adjacent to the lumen) as a percentage of total airway cells. 8 airways from n=3 mice per group were analysed.
**Allergen sensitization**

8-10 week old mice (no more that 1 week difference in age within any one experiment) were dosed with purified HDM extract (Greer Laboratories, NC, USA, batch number 7500: 21.26μg derP/mg protein; 13.55 endotoxin U/mg) intranasally (1μg/μl in 25μl Phosphate-buffered saline (PBS) 3 times/week for 3 weeks, littermate control mice received PBS only. Lung function testing was carried out 24hr following the last dose.

**Measurements of airway hyperresponsiveness (AHR)**

Unrestrained conscious mice (male and female) were placed into a plethysmograph chamber (Buxco Europe Ltd., Hampshire, UK). Airway resistance was measured as Penh (enhanced pause) (17). Baseline Penh was measured for 5 min, after which time an aerosol of methacholine (Sigma-Aldrich, Dorset, UK) was given and Penh was again measured for 5 minutes. The change in Penh was calculated between baseline and each subsequent methacholine dose.

Respiratory mechanics were also directly measured using the forced oscillation technique (Flexivent system, SCIREQ) in anaesthetized, ventilated female mice 24 hours post-challenge. Following lung function measurements serum, BALF and lung tissue samples were collected and processed based on the method by Hamelmann et al (18). BALF was collected using 3 x 0.4ml sterile PBS, and centrifuged. The supernatant was stored for further analysis, and the cell pellet was re-suspended in 0.5ml RMPI supplemented with 10% FBS, penicillin and streptomycin. 50,000 cells were spun at 400 rpm for 4 min using a Cytospin3 (Shandon), and fixed in methanol for 5 min. Cells were stained using Wright-Giemsa stain (Sigma-Aldrich) following manufacturer’s instructions. Eosinophil percentages were obtained by determining the number of eosinophils in a sample of 300 cells per mouse. Left lung lobes were inflated and fixed for histology as detailed above.

**Quantification of peribronchiolar collagen and goblet cell counts in lung tissue samples**

To quantify collagen around the airways, 4 μm wax sections were stained with Sirius red. Digital images were obtained from airways that were similar in size using a 40x lens under polarised light. All images were obtained in one sitting to minimise any
variability. For each image, a threshold was used so only the Sirius red staining was highlighted, and the integrated density of staining around each airway was determined. Image analysis was performed using Fiji software. For each mouse four airways were measured and the mean average was calculated.

Goblet cells counts were obtained using the protocol described in Townsend et al. (19). Periodic Acid-Schiff (PAS)-stained goblet cells in airway epithelium were counted in 4 µm wax lung sections. Counting was undertaken blind using a numerical scoring system (0: 5% goblet cells; 1: 5 to 25%; 2: 25 to 50%; 3: 50 to 75%; 4: >75%) The sum of airway scores from each lung was divided by the number of airways examined, 20–50 per mouse, and expressed as mucus cell score in arbitrary units (U).

**Cell culture of human bronchial airway epithelial cells**

Bronchial epithelial transformed cells (BEAS-2B) were purchased from LGC standards (Teddington, UK) no contamination was found in the cell stock. The cells were cultured in keratinocyte media, supplemented with recombinant human epithelial growth factor (rhEGF) and bovine pituitary extract (BPE) (all Gibco, Paisley, UK) as previously described (20). Following overnight culture in minimal media to enable synchronization, cells were stimulated with IL-1β (10ng/ml, Sigma Aldrich) or dexamethasone (Sigma Aldrich) and proteins isolated at 2h or 24h for analysis of supernatants by ELISA. Proteins were extracted from cells using a nuclear extraction kit (Active Motif, Rixensaart, Belgium) following manufacturers instructions.

**Knockdown and overexpression of DPP10 in BEAS2B cells**

*DPP10* gene expression was knocked down using 50nM ON-TARGET plus SMART pool siRNA (Thermo-Scientific Dharmacon, CO, USA) against the *DPP10* gene as described previously (20). Silencer select siRNA (Ambion, TX, USA) was used as a control for transfection. The DPP10 clone 11 (1) was overexpressed using jetPEI (Polyplus-transfection, Illkirch, France) according to the manufacturer’s instructions.
**Knockdown of DPP10 in NHBE cells**

Primary human bronchial epithelial (NHBE) cells were obtained from Lonza and no contamination was found in the cell stock. The cells were cultured to a maximum of five passages to limit variable responses in bronchial epithelial medium (hAEC Culture Medium . Epithelix). Cells were seeded into 24-well plates (Corning Costar Corp.) at 4x10^4 cells/well to reach 40–60% confluence on the day of transfection. RNA interference (RNAi) was carried using 150nM ON-TARGETplus SMARTpool (Dharmacon Research Inc., Lafayette, CO) or ON-TARGETplus Non-targeting Pool negative control. RNAi-complexes were added to the cells were placed in a CO2 incubator at 37°C for 48 hours. Following transfection, culture medium was changed and then stimulated with 1 ng/ml of IL-1β. Cells Supernatants were collected at different time points post-stimulation (0h, 4h, 10h, 16h, 24h and 30h) for cytokines measurement. qPCR primer sequences were as follows: DPP10 forward primer GTGAAGGTCCAAGGGTC; reverse primer CTGGCTTTCCTATCTTCTTC. GAPDH forward primer: TCAAGAAGGTGGTGGTGAAGCAG; reverse primer CGCTGTTGAAGTCAGAGGAG.

**Enzyme-linked immunosorbent assays (ELISA)**

Levels of Secretory Leukocyte Protease Inhibitor (SLPI) in the supernatant, were measured by ELISA following manufacturer’s instructions (R&D systems, Minneapolis, MN, USA), 24 hours following cell stimulation. Human IL-6 and IL-8 were measured by ELISA DuoSet (R&D Systems Europe; Abingdon, Oxon, UK), according to the manufacturer’s instructions. Mouse serum IgE levels were measured by ELISA assay using purified anti-mouse IgE capture antibody (BD Pharmingen Cat No 553413), according to the manufacturer’s instructions.

**Western Blotting**

Western Blotting was performed as described previously (20) using an anti-glucocorticoid receptor (GR) antibody (H-300, Santa Cruz Biotechnology), 1:1000 and visualised using Luminata™ Forte solution (Millipore, Billarica, MA USA) with exposure to X-ray film (Fisher Scientific, Loughborough, UK).
**Statistical analysis**

Graphs were generated using GraphPad Prism software (GraphPad version 5.0, La Jolla, CA, USA) or Microsoft Excel. Where appropriate, sample sizes were determined using power calculations based on previous experimental data. Data are expressed as mean ± standard error of Mean (SEM). Data on resistance, elastance and compliance at 100mg/ml of methacholine and ΔPenh at 50 mg/ml methacoline are expressed as box and whisker plots showing median and interquartile range. For house dust mite challenge experiments, statistical significance of HDM-treated vs PBS control groups in wild-type and Dpp10^145D^ mice was determined by a two-tailed P value using the Mann–Whitney U test when comparing between two groups only. For knockdown experiments in human cells, graphs show mean values ± SEM of SLPI, IL-6 and IL-8 between DPP10 knockdown cells and control cells. Differences between groups were tested using a two-tailed Student's t-test. A P-value of < 0.05 was considered significant for all statistical analyses.

**RESULTS**

**Mouse Dpp10 genomic structure**

We identified the full-length mouse cDNA of Dpp10 and aligned this with available information on Dpp10. Mouse genomic Dpp10 is located on chromosome 1 and has 26 exons encoding 796 amino acids. The key domains found within the sequence are a trans-membrane domain and a β-propeller region (Supplemental figure 1). We therefore screened the ENU DNA archive for mutations within exons encoding either of these domains.

**Identifying and validating ENU mutations in Dpp10**

From this initial screening, we identified three mutations in 3480 DNA samples. The only non-synonymous mutation found caused an amino acid change from valine to aspartic acid in exon 5 (Figure 1A, B). The valine, or at least a hydrophobic residue at this position, is well conserved in DPP10 and DPP6 orthologous sequences throughout vertebrates (Figure 1C). The wider family of DPPIV domain containing proteins do not generally conserve this position, but aspartic acid (D) has not been
found in any of the proteins defined by the Protein FAMILies database of alignments and Hidden Markov Models (PFAM) profile (21). The mutated residue is on the surface of the protein at the entrance to a pocket in the centre of the β-propeller region. A surface exposed hydrophobic residue in such a position may well be involved in determining substrate specificity and therefore a mutation in this position is highly likely to have some functional impact on DPP10.

**Recovery and maintenance of Dpp10 mutant mice**

To determine whether the mutation identified would have an impact on DPP10 function in vivo, we established a mouse line carrying this mutation, using the corresponding F1 male sperm sample from the Harwell DNA archive (14). In vitro fertilization with the mutagenized sperm was performed using C3H embryos to facilitate genotyping of the mutation that had been induced in Balb/C mice. We established a genotyping strategy using an enzymatic diagnostic digest with HpyChe4IV, followed by PCR to distinguish mutant mice from wild-type littermates (Figure 1D). Seven offspring were obtained, six of which contained the mutant allele indicating that a single copy of the mutation did not affect mouse viability or fertility. We subsequently established a congenic Dpp10 line by backcrossing these mice to CH3 for 10 generations; thereby ensuring that the line did not contain any additional ENU mutations. Dpp10 heterozygotes were intercrossed and the genotypes of their offspring were analysed at E18.5. Normal Mendelian ratios of homozygous, heterozygous and wild-type embryos were found, indicating no prenatal mortality and this was confirmed by Chi squared analysis. Postnatally, Dpp10145D homozygotes and heterozygotes were also recovered in expected numbers and these were morphologically indistinguishable from wild-type littermates. All subsequent experiments were conducted using either homozygous Dpp10 mice or their wild-type littermates.

**Histological examination of Dpp10 mutant mouse lungs**

Dpp10145D homozygotes survived to term and histological analysis did not reveal any visible differences in adult lung architecture (Supplemental figure 2). We investigated the localisation of Dpp10 protein in both wild-type and homozygous Dpp10 mutant lungs by immunostaining. In PBS treated wild-type and Dpp10145D/145D lungs showed a sub-set of airway epithelia cells with Dpp10 positive staining (Figure 2A, B).
Dpp10 staining was much more visible in the airways of both wild-type and mutant mice treated with HDM (Figure 2 C, D). Staining of sections from the same samples shown in C and D with CC10, a Club cell marker, highlights differences in the patterns of Dpp10 and CC10 staining (Figure 2 E, F). The discrepancy is particularly striking in Dpp10 homozygous airways (Figure 2 D vs F). More detailed comparison of Dpp10 localisation in wild-type and Dpp10^{145D} airway cells showed that in wild-type airways, staining was frequently observed at the apical surface of cells whereas in mutant airways, cells with apical Dpp10 staining were much rarer (P < 0.05, Figure 2F). This data indicates that the point mutation in Dpp10^{145D} leads to altered protein localisation rather than complete loss of protein. Further analysis of the lungs showed no visible difference in the cell-type specific markers pro-Surfactant protein-C, Club Cell-10 protein, Smooth muscle actin or Aquaporin-5 between Dpp10 homozygotes and wild-type mice (Supplemental figure 3), indicating that the Dpp10 mutation does not affect cell differentiation.

**The response of Dpp10 mutant mice to house dust mite challenge**

To determine whether the Dpp10^{145D} mutation had a functional impact and in particular, whether the mutation might modify airway hyper-responsiveness, we dosed wild-type (n = 9) and Dpp10 homozygous (n = 10) mice with 25µg house dust mite extract (HDM) 3 times per week for 3 weeks using a previously established protocol (22). Mice were then challenged using a methacholine dose response curve 24 hours after the final HDM dose. Airway responsiveness was initially assessed in a whole-body plethysmograph, using Penh. At baseline and at lower doses of methacholine (12.5 and 25 mg/ml), we found no significant difference in Penh between wild-type and Dpp10 homozygotes (Supplemental Figure 4A), however at the highest dose of Penh tested (50 mg/ml), Dpp10 homozygotes showed a significant increase in Penh (supplemental figure 4B) (P < 0.05).

Following plethysmograph experiments, which indicated increased airway resistance in Dpp10 homozygous mice, we conducted a further HDM challenge prior to directly test respiratory function using the forced oscillation technique (Flexivent). Dpp10 HDM treated mutant mice showed significantly increased airway resistance in response to methacholine challenge (Figure 3A) at 100mg/ml compared to wild-type HDM treated mice (P < 0.05, Figure 3B). Dpp10 HDM treated mutant mice also
showed significantly increased elastance (Figure 3C Vs D, \( P < 0.05 \)) and decreased compliance (Figure 3E Vs F, \( P < 0.05 \)) in response to methacholine challenge at 100mg/ml compared to wild-type HDM treated mice.

To analyse the response of \( Dpp10 \) homozygous mice to allergen challenge further, we assessed a number of hallmarks of allergic airways disease. IgE levels in serum of HDM treated \( Dpp10^{145D} \) mice were significantly higher compared to wild-type HDM treated littermates (Figure 4A, \( P < 0.05 \)). We also found an increased percentage of eosinophils in BALF of \( Dpp10^{145D/145D} \) HDM treated mice compared to the wild-type HDM group (Figure 4B, \( P < 0.05 \)). Quantification of collagen thickness around airways showed a trend towards increased thickness in \( Dpp10^{145D} \) homozygotes, however this was not statistically significant (Figure 4C, D). We did not observe and noticeable difference in goblet cell numbers across all groups of mice (Figure 4E, F).

**DPP10 protein is present in human asthmatic airway epithelial cells and modulation of DPP10 affects key inflammatory mediators**

In order to investigate the possible functional roles of DPP10 in human lungs, we also investigated DPP10 expression in asthma patients. In agreement with our findings in mouse, we found very little DPP10 protein in the bronchial airway epithelium of healthy subjects while in contrast expression was enhanced in epithelium of asthmatic patients (Figure 5A). As with the murine data, DPP10 was only seen in some epithelial cells.

Data from the murine model indicated a potential protective role of DPP10 in the airways so we investigated the effects of DPP10 knockdown and overexpression in human bronchial epithelial cells (BEAS-2B). \( DPP10 \) siRNA effectively silenced the expression of DPP10 (Figure 5B). Knockdown of DPP10 enhanced IL-1β-induced leukocyte proteinase inhibitor (SLPI) release from BEAS-2B cells although this did not reach significance (Figure 5C). In contrast, overexpression of DPP10 resulted in a significant reduction in IL-1β-induced SLPI release (Figure 5C). As the functional effect of DPP10 in vivo suggested a possible effect on endogenous anti-inflammatory corticosteroid production, we examined the effect of \( DPP10 \) modulation on the glucocorticoid receptor (GR). DPP10 knockdown attenuated the ability of GR to translocate to the nucleus and bind to DNA whereas DPP10
overexpression significantly enhanced GR activation even without dexamethasone treatment (Figure 5D). This data was confirmed by Western blot analysis of GR nuclear translocation in BEAS-2B cells (Figure 5D lower panels).

To further investigate whether DPP10 could modulate cytokine release, we also conducted siRNA mediated knockdown of DPP10 in primary epithelial cells (Normal Human Bronchial Epithelial Cells, NHBE). Knockdown with 150nM DPP10 siRNA for 48hr resulted in a 66 percent reduction in DPP10 transcript levels, compared to control. Following siRNA knockdown, NHBE cells were stimulated with IL1β and the levels of IL8 (Figure 5E) and IL-6 (Figure 5F) released from control and DPP10 knockdown cells were measured at 0, 4, 10, 16, 24, and 30 hours following stimulation. DPP10 knockdown increased the level of IL-8 and IL-6 released from NHBE cells at 24 and 30 hours (the increase was statistically significant for IL-8 but not IL-6). These results are consistent with the idea that Dpp10 plays a protective role in asthma.

**DISCUSSION**

Using a Dpp10 point mutant, with a valine to aspartic acid change in the β propeller region, we demonstrated that homozygous mutant mice exhibit greater AHR following HDM challenge than wild-type animals. We also find that DPP10 protein is present in airway epithelial cells and Dpp10 immunostaining is increased in mice following HDM challenge; this corresponds with clinical findings where asthmatic patients show increased DPP10 in lungs compared to controls. Modulation of DPP10 in BEAS2B and NHBE cells resulted in altered levels of cytokine release following IL-1β stimulation as well as modified glucocorticoid function. These results provide biological evidence to support previous findings from human genetic studies indicating that DPP10 is an asthma susceptibility gene.

Positional cloning and Genome wide association studies (GWAS) have identified several genes that are associated with asthma. Understanding the functions of these genes is an important next step to shed light on the aetiology of asthma. This is particularly important for genes like Dpp10, where very little is known about their biological function in the lungs (23). The association of DPP10 with asthma has been confirmed across several different ethnic populations (2-6). In addition, recent studies have shown that DPP10 genetic variants may affect lung function decline in aging
DPP10 is a potassium channel associated protein (25, 26) but unlike other DPP family members, mammalian DPP10 lacks enzymatic activity and is unable to cleave terminal dipeptides from asthma-related cytokines and chemokines (1). Interestingly however, in *Drosophila* DPP10 acts both as an ion channel substrate and has peptidase activity (27).

DPP proteins contain a β-propeller domain, which regulates substrate access to an α/β hydrolase catalytic domain. Most interactions of DPP10 with other proteins are likely to occur on the β-propeller domain so it is significant that the mutation in *Dpp10*^145D^ is in this domain (25). In the brain, DPP10 malfunction is associated with neurodegenerative conditions like Alzheimer and fronto-temporal lobe dementia. Moreover, *DPP10* variants in neurons have been shown to alter potassium channel-gating kinetics (28) and additional studies have shown that DPP10 modulates the electrophysiological properties, cell-surface expression and subcellular localization of voltage-gated potassium channels (29). Given the location of the mutation in the *Dpp10*^145D^ mouse line, it is tempting to speculate that ion channel gating kinetics may also be altered in the lungs of these mice.

Like human *DPP10*, mouse *Dpp10* has 26 exons and multiple splice variants, which retain the transmembrane domain and β-propeller domains. Mouse ENU mutagenesis has proved to be a powerful tool for studies of human diseases, particularly prior to the discovery of Crispr/Cas9 gene editing techniques. Through backcrossing, mice harbouring the selected mutation on a congenic background can be obtained (30). In the current experiment, we identified one non-synonymous mutation, resulting in an amino acid change from valine to aspartic acid in the β-propeller domain of *Dpp10*. Careful comparison of embryonic *Dpp10* homozygotes and wild-type littermates did not reveal any visible phenotype indicating the *Dpp10*^145D^ mutation does not affect the development of mouse lungs.

In this study, we found that mouse DPP10 is localized to the airway epithelium, and more Dpp10 is visible in airways, after HDM treatment. This finding is consistent with a previous report showing Dpp10 protein in the airway epithelium of rat lungs (9). Comparison of airways stained for Dpp10 and CC10, a marker of Club cells, indicates that not all Club cells are positive for Dpp10, since CC10 stained cells are more prevalent than Dpp10 positive cells. Our data does indicate that at least
some CC10 positive cells are also positive for Dpp10. However, further investigation with additional cell-type specific markers will be required to determine which subtype(s) of airway epithelial cells express Dpp10 e.g. Basal cells, Ciliated cells etc. Comparison of the sub-cellular pattern of CC10 staining with that of DPP10 did reveal differences in localisation of these two proteins. Moreover, the localisation of Dpp10 protein is different in airways of wild-type and Dpp10<sup>145D</sup>; there are significantly fewer cells with Dpp10 at the apical surface in mutant lungs. These observations indicate that loss of function in the Dpp10<sup>145D</sup> mice is likely to result from altered protein function rather than lack of protein. This is a frequent consequence of point mutations in both mouse models and in the human population (31, 32).

HDM is one of the commonest aeroallergens worldwide and up to 85% of asthmatics are typically HDM allergic. Inhalation of HDM by naïve mice results in lung function changes including increased resistance and reduced compliance as well as recruitment of inflammatory leukocytes (22, 33). HDM challenge initiates a Th2-polarized response with T2 helper cytokines being produced in both BALF and lungs of mice, including IL-4, 5 and 13 and typically an influx of eosinophils and increase in IgE levels. This cytokine response is important driving the development of airway remodelling which occurs after the Th2 cytokine response and altered lung function (22, 34).

Our results show that after HDM challenge, Dpp10 homozygotes display significantly increased AHR upon methacholine challenge compared to wild-type littermates. Specifically resistance and elastance are increased and compliance is reduced. Following HDM treatment, serum IgE levels and eosinophil counts in BALF were significantly higher in Dpp10 mutants after HDM treatment. Although collagen thickness and goblet cells were not significantly increased, it is known that these hallmarks of airway remodelling only become significantly altered after a longer and more frequent HDM dosing regimen than the one used here (22, 33, 35).

In asthmatics, exposure to the allergen HDM triggers the release of cytokines as a result of mite-derived protease activity (36). Anti-proteases such as SLPI can inhibit this cytokine release. SLPI is an anti-inflammatory gene induced by glucocorticoid in human epithelial cells and low SLPI has been associated with severe asthma in mice and humans (37, 38) In this study we showed that DPP10 overexpression in BEAS2B cells results in lower levels of SLPI release following
cytokine stimulation and enhanced corticosteroid activity. In combination with our finding that DPP10 knockdown in primary human bronchial epithelial cells leads to increased IL-8 and IL-6 release, these data demonstrate that alterations in DPP10 levels can modify the inflammatory response in lung epithelial cells.

In summary, we have established a congenic mouse line harbouring a mutation in the β-propeller domain of DPP10. Our data provide functional experimental evidence supporting previous human genetic data indicating DPP10 is an asthma susceptibility gene. Results from both murine and human in vivo and in vitro analysis suggest that DPP10 may play a protective role in asthma. The novel Dpp10 mouse mutant reported here provides a means to investigate the functional roles of Dpp10 in vivo, for the first time. Understanding how the mutant Dpp10 Kv4 ion channel complex is impacted by the DPP10145D mutation and how this affects other inflammatory associated factors such as TGF-β and IL-33 will be important future studies.

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REFERENCES


Figures

A. Wild type

B. Mutant

C. The alignments of the first β-propeller of DPP10 in seven species

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Mutation: valine (V) to aspartic Acid (D)

D. Genotyping the mutation of Dpp10 allele

Figure 1: The Dpp10 mutation and genotyping in mice

(A) Wild-type sequence for Dpp10. (B) Mutant sequence of Dpp10. (C) Alignment of the sequences containing the first β-propeller of DPP10 across seven species. (D) Gel comparing the genotyping results from wild-type (left lane) and heterozygous (right lane) Dpp145D littermates. Using the following primers:

Forward, AGTCTTGTCTTTACCACA and Reverse, AAGCCTCCAGACACTCAC, PCR generated a 194 bp product. Restriction enzyme digest with HPYChe4IV cut the wild-type allele at ACGT producing two bands of 127bp and 67 bp, whilst the mutant allele remained uncut (194 bp). WT: Wild-type mouse, M: mutant mouse.
Figure 2: Dpp10 localization in mouse airways

Dpp10 positive staining is present in some airway epithelial cells in both wild-type (A) and homozygous (B) lungs, red arrows indicate examples of positively stained cells. Treatment with house dust mite (HDM) resulted in increased DPP10 staining in both genotypes (C), (D). In wild-type mice Dpp10 positive staining was frequently present at the apical surface of airway epithelial cells (blue asterisks) (C). In Dpp10^{145D} homozygotes, few cells with apical Dpp10 staining were present, in contrast, strong staining was frequently observed more basally (black asterisks) (D). Many cells in both WT (E) and Dpp10^{145D} homozygous airways (F) are positive for the Club cell marker CC10. Haemotoxylin stained control airways with primary antibody omitted (E). Quantification of cells with apical Dpp10 staining from 8 airways in N= 3 mice per group (F), mean ± SEM, Student’s t-test, P < 0.001 for WT vs Dpp10^{145D} PBS treated mice and P<0.05 for WT vs Dpp10^{145D} HDM treated mice. Scale bar = 12.5µM. a = airway lumen,
Figure 3: Following House Dust Mite challenge, Dpp10 homozygous mice show increased airway hyperresponsiveness compared to wild-type littermates.

Lung function was measured using the FlexiVent system. The effect of methacholine on airway resistance (A, B), airway elastance (C, D) and airway compliance (E, F) is shown. Dose response curves are shown in (A), (C) and (E), mean ± SEM. Airway resistance (B), airway elastance (D) and airway compliance (F) at 100 mg/ml methacholine shown in detail. Box and whisker plots depict the median and interquartile range (IQR). N = 4 mice for PBS groups, N = 6 for HDM groups, Mann-Whitney U test * P < 0.05.
Figure 4: *Dpp10* homozygous mice develop allergic inflammation in the lungs after house dust mite (HDM) challenge

Exposure of *Dpp10* homozygotes to HDM resulted in enhanced serum IgE levels (A) and elevated eosinophil counts in bronchoalveolar lavage fluid (BALF) (B) compared to wild-type littermates. *Dpp10* homozygotes showed a non-significant trend towards increased peribronchiolar collagen (C, D) and goblet cell numbers compared to wild-type littermates (E, F). Data represented as mean ± SEM, N=4 mice for all groups of IgE (A); eosinophil percentage N=4 for WT PBS, WT HDM and *Dpp10* PBS groups, N=6 for *Dpp10* HDM group (B). Sirius Red N=4 for WT PBS, *Dpp10* PBS and *Dpp10* HDM groups, N=6 for WT HDM group (C). Goblet cell numbers N=3 for WT
and Dpp10 HDM groups (E). Mann-Whitney U test * P < 0.05; *** P < 0.001. Scale bars: D=12.5μM, F=25μM.
Figure 5: DPP10 is increased in human asthmatic airways and modulates release of Inflammatory mediators from human airway epithelial cells

Representative immunohistochemical staining of bronchial biopsies from asthmatic and normal healthy subjects are shown in (A). Results are representative of samples from 3 subjects. A concentration-dependent reduction in DPP10 protein expression after 24hrs in BEAS-2B cells following siRNA knockdown (B). DPP10 overexpression suppresses IL-1β-induced SLPI release from BEAS-2B cells whereas DPP10 knockdown results in an increase in SLPI release (C). DPP10 knockdown also attenuates the ability of the glucocorticoid receptor (GR) to be activated by dexamethasone (D) whereas DPP10 overexpression enhanced GR DNA binding. The
lower panel shows representative Western blots of GR translation. IL8 levels in supernatants from *DPP10* knockdown NHBE cells and control cells after 0 to 30 hours stimulation of IL1β (E). IL6 levels in supernatants from *DPP10* knockdown and control cells NHBE cells after 0 to 30 hours stimulation of IL1β (F). Results are expressed as mean ± SEM of 3 or 4 independent experiments. Students *t*-test, ***P < 0.001*** versus control, ### **P < 0.001**, ## **P < 0.01** and # **P < 0.05** versus stimulated samples.
Supplemental Figures

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LKEEVSLPQLEEPED

TM: trans-membrane domain: AIALIVVVCSLIT
S98: S98 family sequence
The highlighted area was the DNA sequence screened
The underlined area was the β propeller region

Supplemental Fig.S1

Supplemental figure S1. The amino acid sequence and structural features of mouse DPP10.
Supplemental figure S2. Lung histology of wild-type and \textit{DPP10}\textsuperscript{145D/145D} littermates.

H&E staining of mouse airways (A, C) following WT PBS, (E, G) WT HDM, (B, D) \textit{Dpp10145D} homozygous PBS, (F, H) \textit{Dpp10145D} homozygous HDM treatments.

Scale bars: A, B, E, F 25\(\mu\)M, C, D, G, H 12.5\(\mu\)M. a = airway lumen.
Supplemental Fig. S3
Supplemental figure S3. Comparison of cell-type specific markers in wild-type and Dpp10(T145D/145D) and littermates.

Immunohistochemical staining for (A,B) the type II alveolar epithelial cell marker Pro-surfactant C, (C,D) smooth muscle actin, (E,F) Club Cell 10 for Clara cells and (G,H) aquaporin 5 for type I alveolar epithelial cells. No significant differences were visible between wild-type (A,C,E,G) and homozygous (B,D,F,H) mouse lungs. Control sections were processed simultaneously with A-H but with the primary antibodies omitted, representative examples of control sections for anti-rabbit/mouse universal staining kit (I) and anti-goat staining kit (J) are shown. Scale bars A-H = 12.5 μM.
Supplemental figure S4. Comparison of Penh in wild-type and $Dpp10^{145D/145D}$ littermates following house dust mite challenge

Lung function measured in unrestrained conscious mice placed in a plethysmograph chamber. N=9 for WT and N=10 for $Dpp10^{145D}$. ΔPenh in wild type and $DPP10$ mutant mice in response to increasing doses of methacholine (A). ΔPenh at 50mg/ml methacholine (B). * P < 0.05, determined by Mann–Whitney U test.
Table S1. PCR primer sequences for *Dpp10* exons.

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