**Glycoprotein A33 deficiency: a new mouse model of impaired intestinal epithelial barrier function and inflammatory disease**

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**ABSTRACT**

The cells of the intestinal epithelium provide a selectively permeable barrier between the external environment and internal tissues. The integrity of this barrier is maintained by tight junctions, specialised cell-cell contacts that permit the absorption of water and nutrients while excluding microbes, toxins and dietary antigens. Impairment of intestinal barrier function contributes to multiple gastrointestinal disorders, including food hypersensitivity, inflammatory bowel disease (IBD) and colitis-associated cancer (CAC). Glycoprotein A33 (GPA33) is an intestinal epithelium-specific cell surface marker and member of the CTX group of transmembrane proteins. Roles in cell-cell adhesion have been demonstrated for multiple CTX family members, suggesting a similar function for GPA33 within the gastrointestinal tract. To test a potential requirement for GPA33 in intestinal barrier function, we generated Gpa33−/− mice and subjected them to experimental regimens designed to produce food hypersensitivity, colitis and CAC. Gpa33−/− mice exhibited impaired intestinal barrier function. This was shown by elevated steady-state immunosurveillance in the colonic mucosa and leakiness to oral TRITC-labelled dextran after short-term exposure to dextran sodium sulphate (DSS) to injure the intestinal epithelium. Gpa33−/− mice also exhibited rapid onset and reduced resolution of DSS-induced colitis, and a striking increase in the number of colitis-associated tumours produced by treatment with the colon-specific mutagen azoxymethane (AOM) followed by two cycles of DSS. In contrast, Gpa33+/− mice treated with AOM alone showed no increase in sporadic tumour formation, indicating that their increased tumour susceptibility is dependent on inflammatory stimuli. Finally, Gpa33−/− mice displayed hypersensitivity to food allergens, a common co-morbidity in humans with IBD. We propose that Gpa33−/− mice provide a valuable model to study the mechanisms linking intestinal permeability and multiple inflammatory pathologies. Moreover, this model could facilitate preclinical studies aimed at identifying drugs that restore barrier function.

**KEY WORDS:** GPA33, Intestinal permeability, Colitis, Colorectal cancer, Oral tolerance

**INTRODUCTION**

Impaired intestinal epithelial barrier function is an emerging factor in the aetiology and pathology of a range of gastrointestinal disorders (Turner, 2009), including food hypersensitivity (Ventura et al., 2006), inflammatory bowel disease (IBD) (Heller et al., 2005; Zeissig et al., 2007) and colitis-associated cancer (CAC) (Kim and Kim, 2014). It is becoming clear that gastrointestinal homeostasis depends on the capacity of the epithelial lining to exclude luminal bacterial and food antigens from the lamina propria (LP) and thereby prevent local and systemic inflammation. Accordingly, restoration of barrier function is an important therapeutic ambition for the treatment and prevention of inflammation-mediated pathologies (Fries et al., 2013).

The intestinal barrier is maintained by a number of interacting components: production of mucus and anti-microbial peptides, epithelial integrity, cell-cell adhesion and innate immune responses—defects in any of which can increase intestinal permeability (Pastorelli et al., 2013). Cell-cell adhesion within the intestinal epithelium is regulated by tight junctions (TJs), the most apical of the junction complexes, which block the passage of luminal contents via a paracellular route between cells. TJs are composed of a number of proteins, including occludins, claudins and members of the cortical thymocyte marker for *Xenopus* (CTX) group of type I transmembrane proteins of the immunoglobulin (Ig) superfamily (Chrétien et al., 1998). The CTX group includes members of the junction adhesion molecule (JAM) (Laukoetter et al., 2007; Vetrano and Danese, 2009; Vetrano et al., 2008), the Coxsackie and adenovirus receptor (CAR) (Morton et al., 2013; Rasmperger et al., 2006) and the endothelial cell-selective adhesion molecule (ESAM) (Nasdala et al., 2002) families. Notably, the loss of JAM-A has been implicated in changes to intestinal permeability with subsequent inflammation, cytokine production and colitis in mice (Laukoetter et al., 2007) and IBD in humans (Vetrano and Danese, 2009; Vetrano et al., 2008).

A relatively understudied founding member of the CTX family (Chrétien et al., 1998) is glycoprotein A33 (GPA33) (Heath et al., 1997). In human and mouse, the expression pattern of GPA33 is exquisitely restricted to epithelial cells lining the entire length of the intestine. Within this compartment, GPA33 is present on the basolateral membranes of the epithelial cells of both absorptive and secretory lineages, with notable enrichment of GPA33 protein evident at cell-cell junctions in whole-mount preparations of colonic epithelium (Johnstone et al., 2000). Its robust expression from the...
**TRANSLATIONAL IMPACT**

**Clinical issue**
Intestinal permeability is recognised as an aetiological and pathological factor in a number of intestinal disorders. These include food hypersensitivity, inflammatory bowel disease (IBD) and colitis-associated cancer (CAC), which commonly occur as co-morbidities. The two main forms of IBD, Crohn’s disease (CD) and ulcerative colitis (UC), are chronic conditions that increase the lifetime risk of colorectal cancer development 4–10-fold over the unaffected population and lower the age of onset by about 20 years. At present, anti-inflammatory agents make up the main treatments for IBD. However, restoration of barrier function would be an attractive therapeutic option for the treatment of IBD and prevention of CAC development. To achieve this goal, model systems with impaired intestinal barrier function and susceptibility to food hypersensitivity, in which IBD and CAC can be experimentally induced, are required. Such models offer the potential to study the mechanistic link between intestinal permeability and inflammatory disorders and to test novel therapeutic agents for the restoration of barrier function in multiple disease states.

**Results**
In this study, the authors characterised a knockout mouse with no expression of the intestinal epithelium-specific glycoprotein A33 (GPA33). Gpa33−/− mice exhibit elevated intestinal immunosurveillance in the normal state, indicating a mild increase in intestinal permeability. This is markedly increased by environmental challenge with dextran sulphate sodium (DSS), an intestinal luminal irritant. Using this mouse model, the authors investigated the effect of impaired intestinal barrier function on a range of inducible intestinal pathologies. Gpa33−/− mice exhibited hypersensitivity to food antigens and a markedly increased severity of colitis upon exposure to DSS. Coupled with administration of azoxymethane, a colon-specific mutagen, Gpa33−/− DSS-treated mice developed severe CAC. These observations indicate a fundamental role for impaired barrier function in the susceptibility of Gpa33−/− mice to a range of related inflammatory intestinal pathologies. Moreover, the analysis of publicly available gene expression data revealed that GPA33 RNA expression is reduced in the inflamed bowel of individuals with either CD or UC, suggesting that GPA33 deficiency might contribute to intestinal permeability in human disease.

**Implications and future directions**
This study demonstrates a non-redundant role for GPA33 in the maintenance of intestinal barrier function. Future work will determine whether this function is achieved through interactions of GPA33 with components of junctional complexes that promote cell-cell adhesion in the intestine. The evidence that loss of GPA33 contributes to increased severity of multiple intestinal disorders associated with intestinal permeability advocates Gpa33−/− mice as an advantageous model in which to test novel therapeutics designed to restore barrier function.

base of the crypts to the tips of the villi and along the entire rostrocaudal axis of the intestinal tract established GPA33 as a definitive marker of the intestinal epithelium (Heath et al., 1997; Johnstone et al., 2000). Clinically, GPA33 expression has been observed in over 95% of colorectal cancers (CRCs) and associated metastases (Garinchesa et al., 1996), an observation that has underpinned an ongoing and concerted effort to use radioiodide-bound murine (Welt et al., 1994, 1990) and humanised (huA33 mAb) monoclonal antibodies to target GPA33-expressing tumours (Ciprotti et al., 2014; Infante et al., 2013; Scott et al., 2005; Welt et al., 2003, 1996). A recent clinical study established the efficacy of the huA33 mAb in penetrating CRC metastases (Ciprotti et al., 2014), promoting continued interest in GPA33 as a therapeutic target in antibody-based treatment of late-stage CRC.

Despite the highly tissue-specific expression pattern and associated clinical interest, relatively little is known about the function of GPA33. Insights into its transcriptional regulation (Johnstone et al., 2002; Mao et al., 2003), and implied roles in cell-cell adhesion (Ackerman et al., 2008) and immune signalling (Mallegol et al., 2005, 2007; Van Niel et al., 2003), constitute the most prominent findings to date. In a prior report, conducted with Gpa33−/− mice on a mixed background (C57BL/6×129SvJ), we noticed a mild elevation in spontaneous colonic ulceration and a more severe response to the colonic injury induced by trinitrobenzenesulfonic acid (TNBS), compared to wild-type (WT) mice (Pereira-Fantini et al., 2010).

In this study, we analysed the role of GPA33 in intestinal barrier function using Gpa33−/− mice on a pure C57BL/6 background. Our findings indicate a role for GPA33 in the maintenance of intestinal barrier function and the prevention of associated pathologies such as IBD.

**RESULTS**

**Gpa33−/− mice do not exhibit spontaneous gastrointestinal pathology**
A null allele of Gpa33 was generated by replacing most of the Gpa33 coding sequence with a promoter-less β-geo cassette as described in supplementary material Fig. S1A. Expression of GPA33 was clearly detectable in the intestinal epithelium of WT mice and undetectable in Gpa33−/− mice, as shown by RT-qPCR (supplementary material Fig. S1B) and immunohistochemistry (supplementary material Fig. S1C), confirming the allele as a bona fide null. Gpa33−/− mice were born at the expected frequency, are fertile and have a normal lifespan. Intestinal epithelial cell lineage differentiation (Fig. 1A) and proliferation (Fig. 1B,C) were found to be normal in Gpa33−/− mice and, apart from an extremely mild, progressive colitis with age, which is indistinguishable from that seen in WT, Gpa33−/− mice did not exhibit steady-state colitis (Fig. 1D,E).

**Gpa33−/− mice have impaired intestinal barrier function**
To study the requirement for GPA33 in the maintenance of intestinal barrier function, we delivered TRITC-labelled dextran (4 kDa) to the gastrointestinal tract by gavage and measured its levels in the circulation 4 h later. WT and Gpa33−/− mice did not exhibit significant, detectable intestinal permeability to dextran in the steady-state. To test barrier function under challenge conditions, the experiment was repeated with a cohort of mice that received 2% dextran sodium sulphate (DSS) in the drinking water for 16 h prior to TRITC-dextran gavage (Brandl et al., 2009). This short-term DSS treatment did not cause morphological epithelial damage in the colon of either genotype (Fig. 2B,C). WT mice were unaffected by 2% DSS treatment, in accord with a published report in which effects on intestinal permeability were first observed in WT mice only after 3 days of 5% DSS treatment (Kitajima et al., 1999). In contrast, after DSS treatment, Gpa33−/− mice demonstrated a more than threefold increase in the level of serum TRITC-dextran (Fig. 2A) over WT mice exposed to DSS, indicating a relative loss of barrier function in Gpa33−/− mice compared to WT.

In order to further interrogate steady-state barrier function in Gpa33−/− mice, dendritic cells (DCs) within the colonic LP were sampled by flow cytometry. A range of DC populations were elevated in the colonic LP (Fig. 2E) but not the spleen (supplementary material Fig. S2B) of Gpa33−/− mice, most notably activated DCs and activated CD103+ gut-resident DCs (Fig. 2D,E). Although more total DCs were present in Gpa33−/− mice, the relative activation state of DC populations in the colonic LP were similar in Gpa33−/− and WT mice (Fig. 2D,F). This elevated immunosurveillance phenotype is not dependent on Gpa33
expression in haematopoietic cells: Gpa33 expression was undetectable in 39 blood cell populations encompassing both myeloid and lymphoid lineages (supplementary material Fig. S3). Collectively, these data suggest that Gpa33−/− mice exhibit an intestinal epithelial cell-intrinsic mild and sub-pathological intestinal permeability (IP) defect that sensitises them to intestinal challenge.

**Gpa33−/− mice are susceptible to colitis induction**

To investigate the effect of GPA33 deficiency on susceptibility to colitis induction, we treated Gpa33−/− mice with 2% DSS. Two different DSS treatment regimens were employed to induce acute or chronic colitis. In the acute colitis model, mice were exposed to 2% DSS in the drinking water ad libitum for 104 h and then switched to normal drinking water for a further 88 h (192 h in total). Gpa33−/− mice exhibited a more rapid onset of colitis compared to WT mice, shown by increased weight loss (Fig. 3A), and histological damage (Fig. 3C,D). Maximal colitis in Gpa33−/− mice was observed at 104 h, whereas the colitis score significantly increased between 104 h and 192 h (P<0.01) in WT mice (Fig. 3C,D), demonstrating accelerated onset and progression of DSS-induced colitis in Gpa33-deficient animals. At the 192 h time point, both genotypes exhibited similar colitis severity, suggesting that Gpa33 deficiency mostly affects colitis onset (Fig. 3C,D). In the chronic colitis model, mice were exposed to three cycles of 2% DSS in the drinking water for 5 days followed by normal drinking water for 16 days. Gpa33−/− mice also exhibited increased susceptibility to chronic colitis as shown by more exaggerated weight loss following each cycle of...
DSS (Fig. 3B) and impaired crypt regeneration and resolution of colonic inflammation compared to WT (Fig. 3E,F). Together, these observations demonstrate rapid onset of acute colitis and delayed resolution of chronic colitis in Gpa33−/− mice.

Gpa33−/− mice are susceptible to colitis-associated cancer (CAC)
Chronic colitis is a susceptibility factor for CRC (Eaden et al., 2001; Kim and Chang, 2014). To test whether the rapid induction and impaired resolution of colitis in Gpa33−/− mice leads to increased risk of CRC, we employed the azoxymethane (AOM)/DSS model (Neufert et al., 2007; Wirtz et al., 2007). Mice were injected with the colon-specific mutagen AOM and subjected to two cycles of 2% DSS in drinking water to induce chronic colitis (Fig. 4A). Colon tumour burden was markedly elevated in Gpa33−/− mice compared to WT mice, as shown by endoscopy of the most distal part of the colon in live animals (Fig. 4B,C) and by quantitating the number and area of the tumours at the end of the experiment (Fig. 4D-F). Both WT and Gpa33−/− mice exhibited activation of the WNT pathway, shown by elevated β-catenin protein (supplementary material Fig. S4A) and upregulated expression of the WNT target genes, CD44 and Lgr5, in tumours compared to normal epithelium (supplementary material Fig. S4B), consistent with CTNNB (encoding β-catenin) being a known AOM target.

Gpa33−/− mice are not predisposed to sporadic CRC
To investigate the requirement for inflammation in the susceptibility of Gpa33−/− mice to CAC, a model of sporadic CRC was employed. Mice were injected weekly with AOM alone (10 mg/kg) for 6 weeks and aged until 18 weeks to allow outgrowth of induced lesions. In
Fig. 3. Gpa33−/− mice are more susceptible to DSS-induced colitis. (A,B) Black bars indicate cycles of 2% DSS treatment provided ad libitum in drinking water. (A) Percentage body weight changes during acute colitis reveal that Gpa33−/− mice lose significantly more weight than WT mice in response to treatment with 2% DSS (asterisks) for 104 h. (B) Percentage body weight changes during chronic colitis reveal that Gpa33−/− mice lose significantly more weight than WT mice after each cycle of DSS treatment, indicated by asterisks on days 8, 31, 50 and 61. Gpa33−/− mice also exhibited significantly reduced percentage body weight on days 10, 12, 47, 51, 52 and 54 (*P<0.05). (C) Representative images of H&E-stained distal colon sections from mice that were exposed to acute DSS treatment for 0, 48 and 104 h and after an additional 88 h of normal water (192 h time-point). Crypt degradation is evident in Gpa33−/− mice at 48 h but not in WT mice until 104 h (brackets). Arrows indicate immune cell infiltration and thickening of the LP, which is first conspicuous at 48 h in Gpa33−/− mice but not until 104 h in WT controls. (D) Histology colitis scores at 0, 48, 104 and 192 h show accelerated colitis onset in Gpa33−/− mice. Also, the histology colitis score is significantly higher at 192 h than at 104 h in WT mice (P<0.01). (E) Representative images of H&E-stained distal colon sections from mice (day 66) that underwent chronic DSS treatment. Arrows indicate immune cell infiltration and thickening of the LP, which is most pronounced in the Gpa33−/− mice. (F) Histology colitis score of mice at day 66 following chronic colitis regimen. Mean±s.e.m. for A,B,D,F; n=5 (A), n=5-8 (B), n=3 (D), n=4 (F); *P<0.05, **P<0.01. Scale bars: 50 µm.
this model, the absence of DSS removes the major inflammatory driver of tumorigenesis present in the AOM/DSS CAC model. *Gpa33*−/− mice did not exhibit an increase in sporadic CRC incidence (Fig. 5A) or tumour size (Fig. 5B,C) compared to WT controls. This finding indicates that inflammation is required for the increased susceptibility of *Gpa33*−/− mice to colonic tumorigenesis.

*Gpa33*−/− mice exhibit increased sensitivity to food antigen

A clinical consequence of increased IP is the loss of tolerance to food antigens, with increasing severity of hypersensitivity correlated with diminishing barrier function (Ventura et al., 2006). To examine delayed-type hypersensitivity (DTH) to oral ovalbumin (OVA) in *Gpa33*−/− and WT controls, mice were gavaged with OVA to induce tolerance, injected intraperitoneally 7 days later with OVA and Freund’s complete adjuvant to boost any primed immune response, and injected with OVA in the ear to elicit local inflammation and changes in ear thickness (Wang et al., 2007). Ear thickness was measured with a calliper at 0, 24 and 48 h after intra-ear injection of OVA. In the positive control cohorts, which were gavaged with PBS alone, or OVA in combination with cholera toxin (OVA+CT) to break tolerance to OVA, topical injection of OVA caused an increase in ear thickness 24 and 48 h later. The
development of DTH and broken oral tolerance in these mice occurred irrespective of genotype, as expected (Fig. 6). For WT mice that had been gavaged with OVA alone, the mean ear thickness was significantly less than that of the PBS and OVA+CT controls, indicating that oral tolerance had been established. In contrast, the mean ear thickness in Gpa33−/− mice that had been gavaged with OVA alone was not significantly different from that of Gpa33−/− mice that had been gavaged with PBS, or OVA+CT, indicating systemic hypersensitivity to OVA and broken oral tolerance (Fig. 6).

**GPA33 expression is reduced in the inflamed bowel of individuals with IBD**

In order to investigate the potential role of GPA33 deficiency in human IBD, GPA33 expression was analysed in two published human IBD microarray datasets (Noble et al., 2008; Olsen et al., 2009). In individuals with either Crohn’s disease (CD) or ulcerative colitis (UC), mean GPA33 expression was reduced in inflamed bowel compared to uninfammed and normal control tissue, respectively (Fig. 7). To address the possibility that the reduction in the mean level of GPA33 expression is related to the epithelial cell content of the inflamed samples, we also analysed the expression of EPCAM, an epithelial marker that is also highly expressed in intestinal epithelium. We found that EPCAM was not differentially expressed between uninflammed and inflamed tissue in both the CD and UC datasets (supplementary material Fig. S5A,B). We also analysed the expression of FAP (encoding fibroblast activation protein, expressed in activated stromal fibroblasts of epithelial cancers and healing wounds) and found that its expression was unchanged between uninflammed and inflamed tissues from individuals with CD and elevated in the inflamed tissues of individuals with UC (supplementary material Fig. S5C,D). Analysis of the expression of an additional epithelial marker (CDHI, encoding E-cadherin) and another stromal marker (VIM, encoding vimentin) reiterated the EPCAM and FAP results, respectively (not shown). Collectively, these data suggest that the observed reduction in mean GPA33 expression in the inflamed tissue of individuals with CD or UC is not simply due to reduced epithelial cell content of the samples. These data suggest that GPA33 deficiency correlates with intestinal inflammation in IBD and that it might contribute to IP in this context.

**DISCUSSION**

Gpa33−/− mice on a pure C57BL/6 background are healthy and do not develop spontaneous colitis or any other gastrointestinal pathology up to 1 year of age. Strikingly, however, Gpa33−/− mice are more susceptible than WT to DSS-induced loss of intestinal barrier function and severe colitis and, when DSS treatment is coupled with prior administration of the mutagen AOM (10 mg/kg body weight) six times at weekly intervals to induce sporadic CRC. (A) Tumour number and (B) tumour area per mouse colon at collection was similar in Gpa33−/− and WT mice. (C) Size distribution of sporadic CRC tumours across all Gpa33−/− and WT mice at the end of the experiment. Mean±s.e.m.; n=6-9.

**Fig. 5. Gpa33−/− mice are not prone to sporadic CRC.** Mice were injected with AOM (10 mg/kg body weight) six times at weekly intervals to induce sporadic CRC. (A) Tumour number and (B) tumour area per mouse colon at collection was similar in Gpa33−/− and WT mice. (C) Size distribution of sporadic CRC tumours across all Gpa33−/− and WT mice at the end of the experiment. Mean±s.e.m.; n=6-9.

**Fig. 6. Gpa33−/− mice lack tolerance to oral antigen.** Gpa33−/− mice exhibit lack of oral tolerance to OVA administered by gavage, with resultant systemic delayed-type hypersensitivity (DTH) response manifested as increased ear thickness at the topical OVA injection site. Administration by gavage of PBS, or OVA in combination with cholera toxin (OVA+CT), make up the positive controls for lack of oral tolerance. Whereas WT mice gavaged with OVA alone exhibit oral tolerance compared to WT mice treated with PBS and OVA+CT (mean±s.d., n=6-7, **P<0.01), Gpa33−/− mice gavaged with OVA exhibit increased ear thickness comparable to positive controls, indicative of lack of oral tolerance and systemic DTH. Compared to baseline levels of ear thickness at the time of local OVA injection (0 h), the percentage increases in ear thickness of Gpa33−/− mice at 24 h and 48 h are 33% (P<0.0001) and 41% (P<0.0001), respectively.

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AOM, they develop significantly more and larger colon tumours. Further evidence that GPA33 plays a role in barrier function is the observation that Gpa33−/− mice fail to establish tolerance to the oral antigen OVA and develop DTH.

To investigate the contribution of GPA33 to intestinal barrier integrity, we assessed immunosurveillance in the colon and paracellular permeation of DSS (2% dextran in WT and Gpa33+/− mice. Although leakiness to dextran was not significantly elevated in Gpa33−/− mice under steady-state conditions, Gpa33+/− mice exhibited a twofold increase in activated CD103+ DCs, the major class of antigen-presenting cells required for induction both of tolerance to benign antigen and inflammation to clear pathogens (Scott et al., 2011). This observation suggests that Gpa33−/− mice exhibit a mild increase in luminal antigen flux into the colonic LP.

To analyse the pathological consequences of increased IP and inflammation on Gpa33−/− mice, we exploited several DSS models of colitis (Wirtz et al., 2007; Wirtz and Neurath, 2007). Short-term DSS treatment (2% DSS for 16 h) reduces mucus protection of the most luminal epithelial cells, and allows penetration of bacteria closer to the epithelium (Johansson et al., 2010). Although this did not affect epithelial cell integrity or induce paracellular permeability in WT mice, which is consistent with previous findings (Kitajima et al., 1999), Gpa33−/− mice exhibited dextran permeability upon mucus layer degradation, indicating an underlying defect in cell-cell adhesion. Similar findings have been reported for mice individually deficient for CAR and Occludin, indicating that a number of junctional proteins might play non-redundant roles in the steady-state maintenance of TJs and barrier function (Pazirandeh et al., 2011; Saitou et al., 2000).

Using a harsher regime of DSS treatment (2% DSS in drinking water for 104 h), Gpa33−/− mice exhibited a rapid onset of acute colitis, indicative of luminal antigen penetration into the LP, most likely caused by TJ impairment and enhanced paracellular permeability. Similar observations have been reported for DSS-treated Jam-A-knockout mice, demonstrating a direct link between TJ dysfunction and severe colitis (Lauketter et al., 2007; Vetrano et al., 2008). When exposed to repeated DSS cycles to induce chronic colitis akin to the flares of inflammation in IBD, Gpa33−/− mice were less able to resolve multiple bursts of epithelial damage and inflammation than WT controls. Considered in conjunction with the reduced GPA33 expression observed in the inflamed bowel of humans with either CD or UC, this suggests a potential role for GPA33 impairment in the progression of IBD.

Individuals with IBD have an increased risk of developing CRC (Eaden et al., 2001; Itzkowitz and Yio, 2004; Kim and Kim, 2014). This fundamental role for inflammation in CRC is modelled using AOM/DSS treatment of mice to induce CAC (Neufert et al., 2007). Strikingly, Gpa33−/− mice develop increased numbers of and larger tumours than WT control mice, suggesting that loss of GPA33 leads to both an increase in the incidence of tumour formation and the rate of tumour growth. These observations are consistent with increased inflammation and cytokine production stimulating outgrowth of AOM-mutated tumour-initiating cells in Gpa33−/− mice (Wirtz et al., 2007). Meanwhile, induction of sporadic CRC in Gpa33−/− mice by injection with AOM alone did not result in elevated tumorigenesis, confirming the essential requirement for inflammation and the underlying loss of permeability as the fundamental defect.

The pathological consequences of IP are not restricted to IBD and localised inflammation in the intestinal tract, but can also lead to loss of oral tolerance and systemic inflammatory effects. A leaky epithelium can allow luminal contents including food antigens to bypass normal antigen processing and initiate an inflammatory response in the LP with consequent systemic sensitisation to that food antigen (Wirtz and Neurath, 2007). Indeed, food hypersensitivity can precede IBD onset and is a recognised co-morbidity (Kraus et al., 2004). Unfavourable and elevated CRC and suggested an epithelial cell-intrinsic requirement for GPA33 in barrier formation and junctional complex integrity (Ackerman et al., 2008). Further work will establish the precise mechanism by which GPA33 acts to maintain barrier function and protect against disease, although the cumulative evidence to date points to a role in cell-cell adhesion.

The highly restricted expression of GPA33 to the intestinal epithelium and CRC is a hallmark that has sustained the interest in GPA33 as a target for antibody-based cancer therapy. Interestingly, however, Sakamoto et al. (2000) also detected weak expression of GPA33 in murine salivary glands. This observation, coupled with two studies regarding Sjögren’s syndrome (SS; Ewert et al., 2010; Nguyen et al., 2008), suggests a possible extra-intestinal role for GPA33 in barrier function. SS is an autoimmune disorder in which the salivary and lacrimal glands are destroyed by infiltrating lymphocytes, leading to symptomatic dry mouth and eyes in affected individuals. Although the aetiology of this disorder is thought to be polygenic, a recent study implicated TJ disruption and resultant pro-inflammatory cytokine exposure within the salivary glands as a key pathogenic factor (Ewert et al., 2010). Interestingly, investigation of SS using inbred mouse models identified Gpa33 as one of a handful of genes deleted in two SS susceptibility loci (Nguyen et al., 2008). This tantalising observation suggests that GPA33 might play a role in barrier function outside the intestine.

A number of genetically modified mouse models with deranged junctional complexes and barrier dysfunction have been reported, for which a subset develop spontaneous or inducible inflammatory...
pathology and provide useful preclinical models of colitis and CAC (Hermiton and Gordon, 1995; Laukoehter et al., 2007; Rudolph et al., 1995; Saitou et al., 2000; Smalley-Freed et al., 2010). The ease with which Gpa33+/− mice can be manipulated to elicit a suite of intestinal pathologies, including loss of oral tolerance, severe colitis and CAC, suggests that this model might provide an attractive option for preclinical studies aimed at identifying drugs capable of restoring intestinal barrier function.

**MATERIALS AND METHODS**

**Animals**
All animal experiments were conducted with the approval of the animal ethics committees of The Ludwig Institute for Cancer Research, The Walter and Eliza Hall Institute of Medical Research and the University of Sydney (Australia). All mice were maintained on a pure C57BL/6 genetic background except those used to assess oral tolerance. Cohorts for the oral tolerance experiments were maintained on a mixed 129/SvJ, C57BL/6 background with littermate controls used at all times. Mice were bred and housed in the same room in a specific pathogen-free facility at the Ludwig Institute of Cancer Research to minimise variation in gut microbiota. All mice were 8 to 12 weeks old at the commencement of treatment, or when analysed, unless otherwise indicated.

**Generation of Gpa33−/− mice**
We generated embryonic stem (ES) cells (129SvJ background) containing a null Gpa33 allele by replacing 20 kb of endogenous Gpa33 sequence from the 5′ end of exon 2 to the 5′ end of exon 7 with a promoter-less targeting vector. The targeting vector contained a translational stop codon (TGA) at codon 35 of exon 2 to terminate translation of an encoded truncated (13 amino acid) GPa33 protein. This was followed by an internal ribosome entry site (IRES) to permit translation of a β-galactosidase-neomycin fusion protein (supplementary material Fig. S1A) to allow selection of targeted neomycin-resistant ES cells and visualisation of Gpa33 promoter activity in vivo via blue staining following X-gal metabolism. A congenic Gpa33+/− C57BL/6 strain was generated by backcrossing the Gpa33−/− 129SvJ×C57BL/6 founder mice to C57BL/6 mice for a minimum of ten generations.

**RNA analysis**
RNA was extracted from purified epithelial cell fractions harvested from freshly dissected small and large intestines (Whitehead et al., 1987) and dissected tumour tissue using TRIzol (Life Technologies, Carlsbad, CA, USA). RNA integrity was analysed on a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and cDNA generated using the High Fidelity cDNA synthesis kit (Applied Biosystems, Waltham, MA, USA). Quantitative RT-PCR was performed using SYBR green (Quantace, London, UK) on an ABI 7300 real-time PCR machine using primers designed to amplify Gpa33, Lgr5, CD44 and Hprt1 (sequences available on request). Expression data was normalised to Hprt1 expression.

**In vivo assessment of intestinal permeability**
Mice were exposed to either normal drinking water or DSS (2% w/v, MW 35-50 kDa; MP Biomedicals, Santa Ana, CA, USA) in drinking water ad libitum for 16 h prior to oral gavage with 200 μl of TRITC-dextran (40 mg/ml in PBS; Sigma-Aldrich, St Louis, MO, USA). Circulating TRITC signal was quantitated by fluorometry on serum collected 4 h post-gavage, as described previously (Brandl et al., 2009).

**Fluorescence-activated cell sorting (FACS) analysis**
Analyses of haematopoietic populations in the colonic LP and spleen were performed, unless otherwise indicated.

**Induction of acute and chronic colitis**
One cycle of colitis was induced by ad libitum exposure to DSS (2%; Sigma-Aldrich, St Louis, MO, USA) in drinking water for 104 h, followed by normal drinking water. In the acute colitis experiment, mice were euthanised after 48 and 104 h of DSS treatment and after a further 88 h of normal water (192 h time-point). To induce chronic colitis, mice were treated with three cycles of 2% DSS interspersed with normal water for 16 days. Mice in both models were weighed daily to observe weight loss consequent to colitis. For histological analyses, the colons were opened lengthwise from anus to caecum, rolled and fixed in 10% neutral buffered formalin (Pathitech, Melbourne, Victoria, Australia).

**Collitis-associated and sporadic colon cancer**
CAC was induced through a single intraperitoneal injection of AOM (10 mg/kg; Sigma-Aldrich, St Louis, MO, USA) in PBS, followed 12 days later by two cycles of DSS treatment. Both cycles comprised ad libitum exposure to 2% DSS for 5 days in drinking water, interspersed with 16 days of normal drinking water. To mimic sporadic CRC, mice received weekly intraperitoneal injections of AOM (10 mg/kg) for 6 weeks. Tumour burden in the colon was monitored using a Coloview high-resolution mouse video endoscopic system (Karl Storz GmbH & Co, Tuttlingen, Germany) as described previously (Becker et al., 2006; Ernst et al., 2015). Tumours were counted and measured in two dimensions with callipers to calculate the area. Prior to fixation, samples of tumour and adjacent normal epithelium were collected for molecular analysis.

**Histological techniques**
Observation of intestinal lineages was performed by staining histological sections (4 μm) with haematoxylin and eosin (H&E; all epithelial cells), phloxine and tartzine (Paneth cells) and Alcian blue Periodic acid-Schiff stain (goblet cells). To detect cells in the S phase of the cell cycle, mice received an intraperitoneal injection of bromodeoxyuridine (BrDU; 100 mg/kg; Sigma-Aldrich, St Louis, MO, USA) 2 h prior to collection of tissues. Visualisation of apoptosis was performed using the Apop-Tag Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore, Billerica, MA, USA) according to the manufacturer’s instructions. Antibodies used to assess cell proliferation (BrDU; BD555624; Life Technologies, Carlsbad,
Oral tolerance to ovalbumin

On day 0 mice were anaesthetised by intraperitoneal injection with Avertin (0.5 mg/g; Sigma-Aldrich, St Louis, MO, USA) and gavaged with 200 µl sodium bicarbonate (0.75%; BDH Chemicals, Radnor, PA, USA). Ear thickness was measured with a calliper by a blinded investigator. Ear thickness was immediately measured with a calliper by a blinded investigator. On day 28, 10 µl of OVA in PBS, or 200 µl ovalbumin (OVA) (100 mg/ml, grade III, Sigma-Aldrich, St Louis, MO, USA) in PBS, or 200 µl OVA in combination with cholera toxin (Sigma-Aldrich, St Louis, MO, USA) was gavaged with 200 µl PBS. Mice were then gavaged with 200 µl PBS, or 200 µl ovalbumin (OVA) (100 mg/ml, grade III, Sigma-Aldrich, St Louis, MO, USA) in PBS, or 200 µl OVA in combination with cholera toxin (Sigma-Aldrich, St Louis, MO, USA). On day 7 all mice were injected subcutaneously with a mixture containing 50 µl OVA (1 mg/ml, grade V, Sigma-Aldrich, St Louis, MO, USA) in PBS and 50 µl Freund’s Complete Adjuvant (Sigma-Aldrich, St Louis, MO, USA). On day 28, 10 µl of OVA (1 mg/ml, grade V) in PBS was injected subcutaneously into the ear of all mice. Ear thickness was immediately measured with a calliper by a blinded observer (t=0 h) and then 24 and 48 h later.

Statistical data analysis

Data are expressed as mean±s.e.m. unless indicated otherwise. P-values were calculated using a two-tailed, unpaired t-test with *P<0.05 considered significant and **P<0.01.

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Competing interests

The authors declare no competing or financial interests.

Author contributions


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Supplementary material

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