Novel roles of Cdx transcription factors in intestinal homeostasis

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Authorization

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// Abstract

The transcription factor Cdx2 is essential for intestinal development and homeostasis. Recent research has demonstrated that Cdx regulates the NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome, a critical driver of inflammation, in agreement with other literature suggesting a role for Cdx2 in inflammatory bowel disease. NLRP3 also induces pyroptosis, an inflammatory form of cell death, via processing of Gasdermin D (GSDMD). However, the effects of Cdx2 on GSDMD, the key executor of pyroptosis, remain undefined. This study investigated the role of Cdx in regulating GSDMD processing necessary to cause pyroptosis in intestinal epithelial cells. Our findings revealed elevated GSDMD cleavage in *Cdx* mutant cells, as well as increased cell death. Inhibition of the NLRP3 inflammasome significantly reduced GSDMD cleavage, consistent with an impact of Cdx on NLRP3-dependent pyroptosis. Additionally, subcellular fractionation and Western blot analyses demonstrated the translocation and oligomerization of cleaved GSDMD from the cytoplasm to the membrane, in *Cdx* mutant cells, indicative of formation of GSDMD pores which cause pyroptosis. These findings are consistent with a role for Cdx2 in regulating pyroptosis in the intestinal epithelium, and highlights the potential of Cdx and GSDMD as biomarkers and therapeutic targets for inflammatory conditions of the intestine such as inflammatory bowel disease (IBD) and colorectal cancer (CRC).

Résumé

Le facteur de transcription Cdx2 est essentiel pour le développement et l'homéostasie intestinaux. Des recherches récentes ont démontré que Cdx régule l'inflammasome NLRP3, un acteur clé de l'inflammation, en accord avec d'autres études suggérant un rôle de Cdx2 dans les maladies inflammatoires de l'intestin. NLRP3 induit également la pyroptose, une forme inflammatoire de mort cellulaire, via le traitement de la Gasdermine D. Cependant, les effets de Cdx2 sur la Gasdermine D, principal exécuteur de la pyroptose, restent indéfinis. Cette étude a investigué le rôle de Cdx dans la régulation du traitement de GSDMD nécessaire à l'induction de la pyroptose dans les cellules épithéliales intestinales. Nos résultats ont révélé une augmentation du clivage de GSDMD dans les cellules mutantes pour *Cdx*, ainsi qu'une augmentation de la mort cellulaire. L'inhibition de l'inflammasome NLRP3 a significativement réduit le clivage de GSDMD, confirmant l'impact de Cdx sur la pyroptose dépendante de NLRP3. De plus, des analyses de fractionnement subcellulaire et de Western blot ont démontré la translocation et l'oligomérisation de la GSDMD clivée, du cytoplasme vers la membrane, dans les cellules mutantes pour *Cdx*, indiquant la formation de pores de GSDMD responsables de la pyroptose. Ces résultats sont cohérents avec un rôle de Cdx2 dans la régulation de la pyroptose au sein de l'épithélium intestinal, et mettent en avant le potentiel de Cdx et de GSDMD en tant que biomarqueurs et cibles thérapeutiques pour les affections inflammatoires de l'intestin, telles que les maladies inflammatoires de l'intestin (MII) et le cancer colorectal (CCR).

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Abbreviations

Abbreviation	Definition
4-OHT	4-Hydroxytamoxifen
7-AAD	7-Aminoactinomycin D
AIM2	Absent in melanoma 2
ALR	AIM2-like receptor
AOM	Azoxymethane
AP	Anterior-Posterior
APC	Adenomatous Polyposis Coli
ASC	Apoptosis-Associated Speck-like protein containing a Caspase Recruitment Domain
ASM	Acid Sphingomyelinase
ATG16L1	Autophagy Related 16 Like 1
ATPase	Adenosine Triphosphatase
Bax	Bcl-2-associated X protein
CAPS	Cryopyrin-associated periodic syndrome
CARD	Caspase Activation and Recruitment Domain
CCL	Chemokine (C-C motif) Ligand
CD	Crohn's disease
CDRE	Cdx Response Element
CDX	Caudal-Related Homeobox
CLDN2	Claudin-2
CRC	Colorectal Cancer

CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR- associated protein 9
CT-GSDMD	C-Terminal Gasdermin D
CXCL	Chemokine (C-X-C motif) Ligand
ChIP	Chromatin Immunoprecipitation
Cre-ERT	Cre recombinase-Estrogen Receptor T fusion protein
DAMP	Damage-Associated Molecular Pattern
DFS	Disease Free Survival
DKO	Double Knockout
DMEM	Dulbecco's Modified Eagle Medium
DNTPase	Deoxynucleotide Triphosphate Triphosphohydrolase
DNase	Deoxyribonuclease
DSF	Disulfiram
DSS	Dextran Sodium Sulfate
DTT	Dithiothreitol
DZ	Dizygotic
E	Embryonic day
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
ERK	Extracellular Signal-Regulated Kinase
ESCRT	Endosomal Sorting Complex Required for Transport
EpCAM	Epithelial Cell Adhesion Molecule
F-actin	Filamentous actin
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum

FBXL2	F-box and leucine-rich repeat protein 2
FSC	Forward Scatter
Flox	Flanked by loxP
GSDMD	Gasdermin D
GSDME	Gasdermin E
GWAS	Genome-Wide Association Studies
Ghrl	Ghrelin And Obestatin Prepropeptide
HNF4α	Hepatocyte nuclear factor 4 alpha
HRP	Horseradish Peroxidase
Hsp90	Heat Shock Protein 90
IAP	Intestinal Alkaline Phosphatase
IBD	Inflammatory Bowel Disease
ICAD	Inhibitor of Caspase-Activated DNase
IEC	Intestinal epithelial Cell
IFABP	Intestinal Fatty Acid-Binding Protein
IFX	Infliximab
IKK	IKappaB Kinase
IL	Interleukin
IL12B	Interleukin 12 subunit B
IL23R	Interleukin 23 Receptor
IκBα	Inhibitor of Kappa B alpha
JAK2	Janus Kinase 2
JNK	c-Jun N-terminal kinase
Ki67	Antigen Kiel 67

LAMC2	Laminin subunit gamma-2
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
Lgr5	Leucine-rich repeat-containing G-protein coupled receptor 5
LoxP	Locus of Crossover in P1
M cells	Microfold cells
MAPL	Mitochondrial-Associated Protein Ligase
MDP	Muramyl Dipeptide
mEGF	Mouse Epidermal Growth Factor
MEP1A	Meprin 1A
MUL1	Mitochondrial Ubiquitin Ligase 1
MZ	Monozygotic
MgCl2	Magnesium Chloride
Muc	Mucin
Musashi1	Musashi RNA-binding protein 1
MyD88	Myeloid Differentiation primary response 88
NF- κB	Nuclear factor-κB
NLR	NOD-like receptor
NLRC4	NLR family CARD domain containing 4
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
NOD2	Nucleotide-binding Oligomerization Domain-containing protein 2
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
NT-GSDMD	N-Terminal Gasdermin D
NT-GSDME	N-Terminal Gasdermin E

Na+/K+ ATPase	Sodium-Potassium Adenosine Triphosphatase
Olfm4	Olfactomedin 4
PAMP	Pathogen-Associated Molecular Pattern
PARP	Poly (ADP-ribose) Polymerase
PBS	Phosphate-Buffered Saline
PE-Cy	Phycoerythrin-Cyanine
PI3K	Phosphoinositide 3-kinase
PRR	Pattern Recognition Receptor
PTEN	Phosphatase and tensin homolog
PTM	Post-Translational Modification
PYD	Pyrin domain
RA	Rheumatoid Arthritis
RIPA	Radioimmunoprecipitation Assay
RLR	RIG-I like receptor
ROS	Reactive Oxygen Species
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
Rel	v-rel avian reticuloendotheliosis viral oncogene homolog
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SENP	SUMO-specific protease
SI	Sucrase-Isomaltase
SLC26A3	Solute Carrier Family 26 Member 3
SNPs	Single Nucleotide Polymorphisms
SSC	Side Scatter

STAT3	Signal transducer and activator of transcription 3
SUMO	Small Ubiquitin-like Modifier
Sox2	SRY-box transcription factor 2
TA	Transit-Amplifying
TAK1	TGF-beta-activated kinase 1
TBST	Tris-Buffered Saline with Tween 20
TEMED	Tetramethylethylenediamine
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF receptor-associated factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRIM	Tripartite Motif-containing protein
Th	T-helper
TrypLE	Trypsin-Like Enzyme
UBC9	Ubiquitin-conjugating enzyme 9
UC	Ulcerative Colitis
Wnt3A	Wnt Family Member 3A
ZO	Zonula Occludens

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Chapter 1

Introduction

Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) is a chronic condition marked by inflammation in the gastrointestinal tract. It affects millions of people globally, significantly impairing their quality of life with symptoms like abdominal pain, diarrhea, weight loss, fatigue, and malnutrition (Baumgart & Sandborn, 2007). In 2023, it is estimated that around 322,600 Canadians were living with IBD, and this number is projected to rise to 470,000 by 2035 (Coward et al., 2023).

IBD includes Crohn's disease (CD) and Ulcerative Colitis (UC). CD is characterized by inflammation that can impact any part of the digestive system but mainly affects the end of the small intestine and the beginning of the colon. The inflammation occurs in a “skip lesion” pattern, where affected areas are separated by healthy tissue segments, and can involve the entire thickness of the intestinal wall (transmural lesions). In contrast, UC is limited to the colon and rectum, with inflammation extending continuously and confined to the superficial layers of the intestinal lining. Chronic inflammation caused by IBD can also lead to DNA damage and create a favorable environment for Colorectal Cancer (Baumgart & Sandborn, 2007). The cytokine TNF-α is a central mediator of inflammation in both UC and CD, playing a pivotal role in the pathogenesis of IBD. Various antagonists targeting TNF-α have been developed, with the first biologic therapy, the anti-TNF antibody infliximab (IFX), approved for CD in 1998 and for UC in 2005 (Vulliemoz et al., 2020). However, while infliximab has transformed IBD treatment, its efficacy is often limited by loss of response, where patients who initially benefit from the therapy develop anti-drug antibodies or experience changes in disease biology over time (Schultheiss

et al., 2021). This challenge highlights the need for a more comprehensive understanding of IBD to address treatment resistance and develop more durable therapeutic options.

The precise cause of IBD remains largely unclear, as complex interactions between genetics and external factors are believed to be involved, contributing to the challenge of fully understanding the disease's etiology. However, many researchers consider genetic predisposition to be one of the most significant factors contributing to its development (Y.-Z. Zhang & Li, 2014).

The genetic contribution to IBD was first highlighted through twin studies, which revealed significantly higher concordance rates in monozygotic (MZ, identical) twins compared to dizygotic (DZ, fraternal) twins. For CD, MZ concordance rates range from 20% to 55%, far exceeding the DZ rates of 0% to 3.6%. Similarly, for UC, concordance rates in MZ twins are between 6.3% and 17%, compared to 0% to 6.3% in DZ twins. These findings emphasize the heritable nature of IBD, particularly in CD, where the genetic influence is more prominent (Gordon et al., 2015).

Recent advances in genetic technologies have significantly enhanced our understanding of IBD, particularly through the use of genome-wide association studies (GWAS). GWAS allow the comparison of the genomes of individuals with IBD to those without to identify genetic variations linked to the disease. These studies focus on single nucleotide polymorphisms (SNPs), which are variations in the DNA sequence associated with disease risk. The SNPs can be located in various genomic regions, including open reading frames, regulatory elements, and intergenic regions, where they can influence gene expression or protein function. Through large-scale analyses, GWAS have uncovered over 240 genetic loci associated with IBD susceptibility (H. Gao et al., 2023; Gordon et al., 2015).

One of the most significant genetic discoveries in IBD research is the *NOD2* gene. *NOD2* is an important component of the immune system, expressed primarily in immune cells such as macrophages,

dendritic cells, as well as Paneth cells in the intestinal lining. It is responsible for recognizing bacterial components such as muramyl dipeptide (MDP), which triggers immune responses (Inohara et al., 2003). Mutations in *NOD2* impair its ability to detect bacterial threats, leading to a diminished initial immune response, allowing bacteria to persist, which can subsequently trigger chronic inflammation in the gut. Additionally, *NOD2* mutations may disrupt the secretion of antimicrobial peptides by Paneth cells, further compromising intestinal barrier integrity and promoting inflammation, particularly in Crohn's disease (Grimm & Pavli, 2004; Y.-Z. Zhang & Li, 2014).

Another gene implicated in IBD, especially Crohn's disease, is *ATG16L1* (Y.-Z. Zhang & Li, 2014). Variants of *ATG16L1* interfere with the process of autophagy, which is critical for removing harmful bacteria from the gut and maintaining immune system balance. Disruptions in autophagy can result in bacterial buildup and chronic inflammation, increasing susceptibility to Crohn's disease. Additionally, *ATG16L1* mutations can impair the function of intestinal epithelial cells and disrupt the secretion of antimicrobial peptides, further compromising the intestinal barrier. These defects in cellular processes contribute to an exaggerated immune response and persistent inflammation in the gut (Khor et al., 2011; Kuballa et al., 2008; Travassos et al., 2010).

The *IL23R* gene plays a key role in IBD. It encodes a receptor for IL-23, a cytokine that helps activate T-helper 17 (Th17) cells, which are important for driving inflammation (Y.-Z. Zhang & Li, 2014). Mutations in *IL23R* can cause the receptor to become overly sensitive, causing the IL-23/IL-17 pathway to become hyperactive. This leads to an increase in Th17 cell activation and the overproduction of pro-inflammatory cytokines, which can worsen inflammation in the gut (Duerr et al., 2006). This pathway is a major contributor to IBD, and mutations in *IL23R*, along with other genes including *IL12B*, *JAK2*, and *STAT3*, are linked to both Crohn's disease and ulcerative colitis (Anderson et al., 2011; Brand, 2009).

The growing number of variant loci associated with IBD underscores the important role that genetic factors play in the disease's development. However, these associated genes explain only 20%-25% of the risk for IBD, leaving much of the basis for the disease unexplained. One possible reason for this could be the presence of rare genetic variations that are more difficult to detect (Zuk et al., 2012). Moreover, some non-heritable factors are thought to contribute to IBD, arising from a complex interaction of elements such as environmental influences, immune system dysfunction, and changes in the gut microbiota (Y.-Z. Zhang & Li, 2014).

Reanalysis of twin studies from Sweden, as well as cohorts from Germany, Denmark, and Norway, indicates that environmental factors may play a more significant role in IBD development than previously recognized (Gordon et al., 2022). Smoking, in particular, has been extensively studied and consistently shown to influence IBD onset by affecting both cellular and humoral immune responses, while also promoting the production of colonic mucus. Furthermore, nicotine, a key component of tobacco, has been found to suppress the activity of Th2 cells and impairs autophagy, a process that is especially important in the context of Crohn's disease. The usage of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) also influences IBD with high dose, prolonged and frequent use being associated with an increased risk of CD and UC (Ananthakrishnan et al., 2012).

Diet and stress are also key factors influencing the development and progression of IBD. One study suggested that artificial food additives commonly found in Western diets could contribute to intestinal inflammation by disrupting the gut barrier function (Dolan & Chang, 2017). Conversely, reducing stress and using antidepressants may help manage IBD symptoms (Goodhand et al., 2012).

The involvement of the immune system in IBD underscores the complex relationship between genetic predisposition and immune dysfunction in the disease's development. For instance, individuals with UC

typically display a Th2-dominant immune response, while those with CD tend to exhibit a more mixed Th1/Th17 response (Zhen & Zhang, 2019). IL-23, a key cytokine, is central to driving these immune responses and plays a role in both innate and adaptive immunity (Sewell & Kaser, 2022). Mutations in immune-regulatory pathways, such as *NOD2* and the IL-23 signaling pathway, play a critical role in the development of IBD, particularly Crohn's disease. *NOD2* mutations impair the immune system's ability to recognize MDP disrupting the regulation of TLR4-mediated responses to lipopolysaccharides (LPS). Normally, *NOD2* provides negative feedback to control excessive TLR4 signaling and inflammation in response to LPS. However, with *NOD2* mutations, this regulation is lost, leading to a weakened initial response to LPS and subsequent over activation of alternative inflammatory pathways, including TLR4 (Kim et al., 2015; Yamamoto & Ma, 2009). Similarly, mutations in the IL-23 pathway contribute to IBD by dysregulating the immune response, demonstrating the interplay of multiple immune pathways in the disease's onset and progression (Korta et al., 2023). These genetic alterations disrupt the normal immune responses to gut microbiota, highlighting how genetic factors contribute to immune system dysfunction in IBD.

While immune dysfunction and genetic factors are central to IBD, it is increasingly recognized that the gut microbiota is also crucial in the disease's development. Several studies have investigated the gut microbiomes of individuals with CD and UC, comparing inflamed and non-inflamed regions of the intestine. These studies have consistently shown that IBD patients have significantly reduced microbial diversity in their fecal microbiomes compared to healthy individuals (Joossens et al., 2011). Furthermore, the microbial composition in individuals with IBD appears to be more unstable compared to those without the disease (Andoh et al., 2011).

In a healthy colon, the mucus layer consists of two distinct layers: the outer layer, which is loosely attached and promotes bacterial growth, and the inner layer, which is tightly adherent and typically

sterile (Y.-Z. Zhang & Li, 2014). In IBD, particularly CD, there is a significant increase in bacteria that adhere to the mucus layer in the colon. This is associated with a decrease in beneficial bacteria such as *Bacteroides*, *Eubacterium*, and *Lactobacillus* species, which play a crucial role in maintaining gut health (Ott et al., 2004). This dysbiosis, or imbalance in gut microbiota, can compromise the integrity of the intestinal barrier by disrupting mucus production, weakening tight junctions between epithelial cells, and increasing gut permeability. These changes allow bacteria and toxins to penetrate the intestine, triggering the chronic inflammation characteristic of IBD and highlighting the critical role of a well-functioning intestinal barrier in gut homeostasis.

All these factors—genetic predisposition, immune system dysfunction, environmental triggers, and gut microbiota imbalances—collide to weaken intestinal defenses and fuel the relentless inflammation that defines IBD, a complex condition (Figure 1).

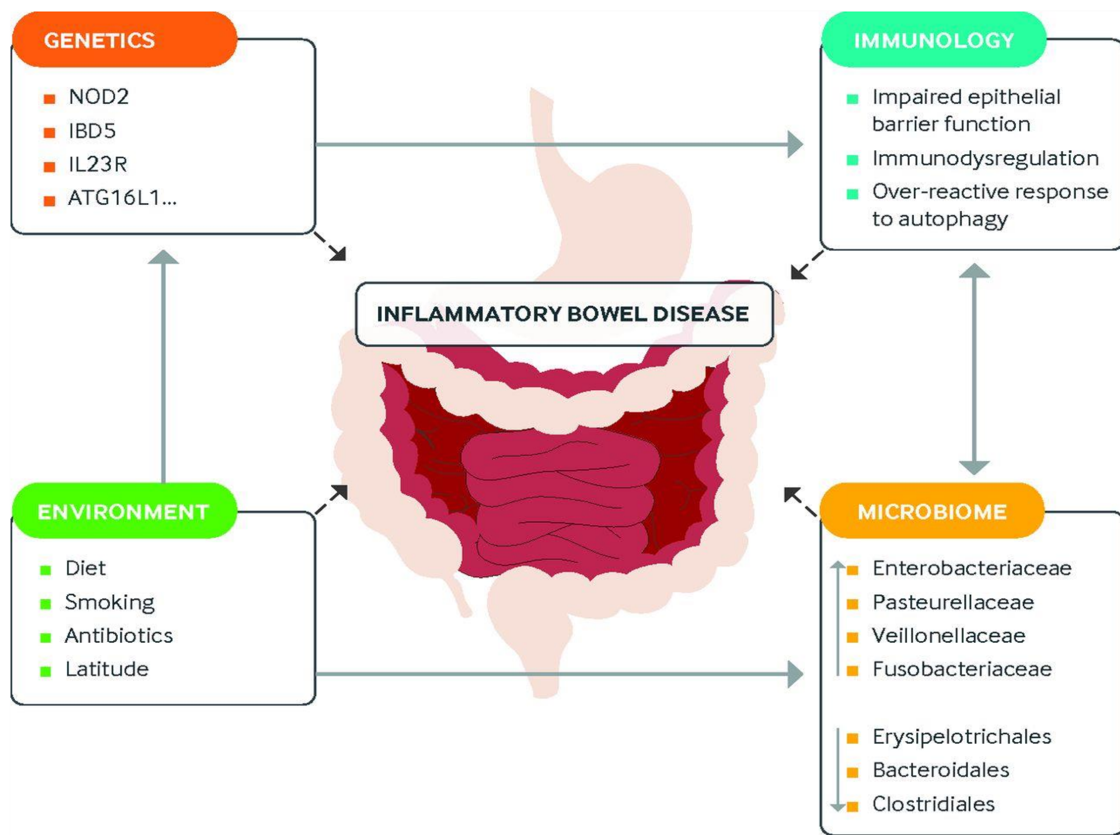


Figure 1: **The etiology of Inflammatory Bowel Disease (IBD).** Although the etiology of IBD remains largely unknown, it is believed to be a combination of genetic susceptibility, environment, intestinal microbial and immune responses (Taken from Oliveira and Monteiro, 2017).

Intestinal epithelium and Cdx transcription factors

The intestine is lined by a specialized single-layered epithelium that facilitates essential processes including digestion and the absorption of water and nutrients, while also serving as a protective barrier against harmful pathogens present in the gut environment. This epithelium stands out as one of the most dynamic and rapidly renewing tissues in adult mammals, ensuring the continuous maintenance of its functions (Heath, 1996).

The epithelial cell types of the small intestine include absorptive enterocytes, Goblet (mucus-secreting) cells, Paneth cells (which secrete antimicrobial peptides to help maintain gut homeostasis), and hormone-secreting enteroendocrine cells (Kong et al., 2018). Stem cells, which reside at the base of the intestinal crypts, are multipotent and self-renewing and are the source of the intestine's various cell types. They divide asymmetrically into a new stem cell and a committed daughter cell known as a transit amplifying (TA) cell, which multiplies a limited number of times before terminally differentiating. Once differentiated, intestinal cells continue an upward migration to reach the villi except for Paneth cells which migrate downward to the base of the crypts (Figure 2) (Lipkin, 1985). The colon has a simple columnar epithelium like the small intestine, but it lacks villi and Paneth cells and contains deeper crypts (Sancho et al., 2004).

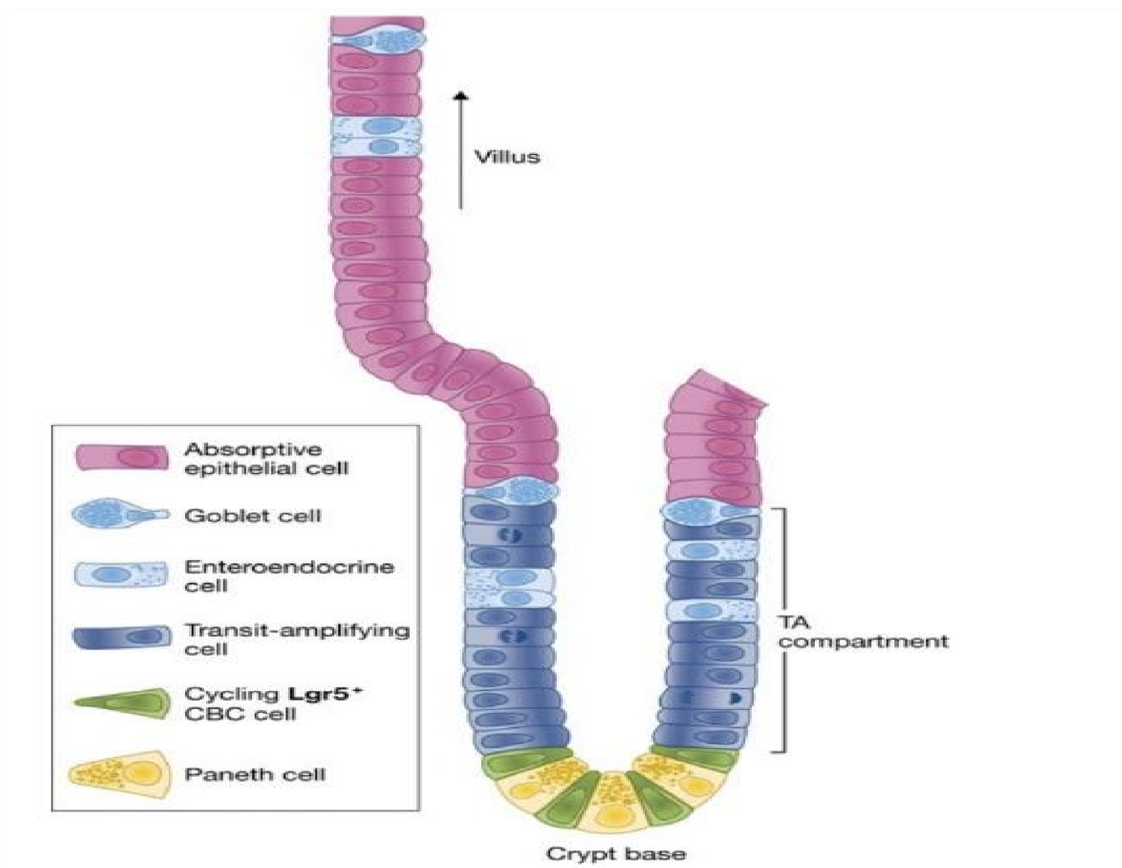


Figure 2: **Schematic of the different cells found in crypt-villous axis in the small intestine.** Stem cells reside at the base of the intestinal crypts and divide to a new stem cell and a transit amplifying (TA) cell. TA cells multiply a limited number of times before terminally differentiating into the various intestinal epithelia cell types (Taken from Schuijers and Clevers, 2012).

The continual renewal of the intestinal epithelium poses significant challenges, as the balance between cell production and cell loss must be tightly regulated. Any disturbance in this equilibrium can weaken the barrier function and potentially lead to the development of intestinal disorders (Vereecke et al., 2011). To maintain this balance, cell proliferation and differentiation in the intestinal epithelium is carefully controlled by a network of transcription factors. Among these, the *Caudal-related homeobox* (*Cdx*) genes stand out as one of the most extensively studied, playing a critical role in ensuring intestinal homeostasis (Coskun, 2014).

The *Cdx* genes, part of the homeobox gene family, are homologs of the *caudal* (Cad) gene in *Drosophila*, and contain a highly conserved sequence encoding the homeodomain, a motif essential for

DNA binding (Banerjee-Basu et al., 2001). This sequence conservation of *Cdx* function is evident across vertebrates, including zebrafish (*Cdx1a*, *Cdx1b*, and *Cdx4*) (Davidson & Zon, 2006), mice (*Cdx1*, *Cdx2*, and *Cdx4*) (Duprey et al., 1988; Gamer & Wright, 1993; James & Kazenwadel, 1991), and humans (*CDX1*, *CDX2*, and *CDX4*) (Bonner et al., 1995; Drummond et al., 1997; Horn & Ashworth, 1995). *Cad/Cdx* genes are crucial for posterior development as well as patterning along the anterior-posterior (AP) axis, and mutations often lead to defects in morphogenesis and organ formation (Macdonald & Struhl, 1986; van Rooijen et al., 2012).

Cdx members regulate target gene expression by binding to specific regulatory sequences known as Cdx response elements (CDREs). These elements typically contain a conserved 5'-TTTATG-3' motif, which is recognized by Cdx proteins which then activate or repress the expression of targets, thus influencing processes like cell differentiation, proliferation, and migration (Guo et al., 2004; James et al., 1994).

The murine *Cdx* genes (*Cdx1*, *Cdx2*, and *Cdx4*) have overlapping patterns of expression in the posterior embryo (Lohnes, 2003). *Cdx1* expression begins at E7.5 in the ectoderm and developing mesoderm of the primitive streak. As the neural ectoderm differentiates, *Cdx1* becomes restricted to the spinal cord region, and by E12, is localized mainly in the tail bud, where it contributes to mesoderm formation. Similarly, *Cdx2* is expressed in the primitive streak and tail bud and plays a role in posterior mesoderm and endoderm formation. *Cdx4* is expressed early in the primitive streak, especially at the caudal end, but diminishes by E10.5 (Figure 3).

Cdx1 and *Cdx2* expression (but not *Cdx4*) persists through later developmental stages, and in the intestinal epithelium in the adult. In the adult intestine, *Cdx1* is expressed in a gradient along the AP axis with a peak in the distal colon, while *Cdx2* expression increases from the duodenum to a maximum in the proximal colon (Figure 4) (Guo et al., 2004; James et al., 1994).

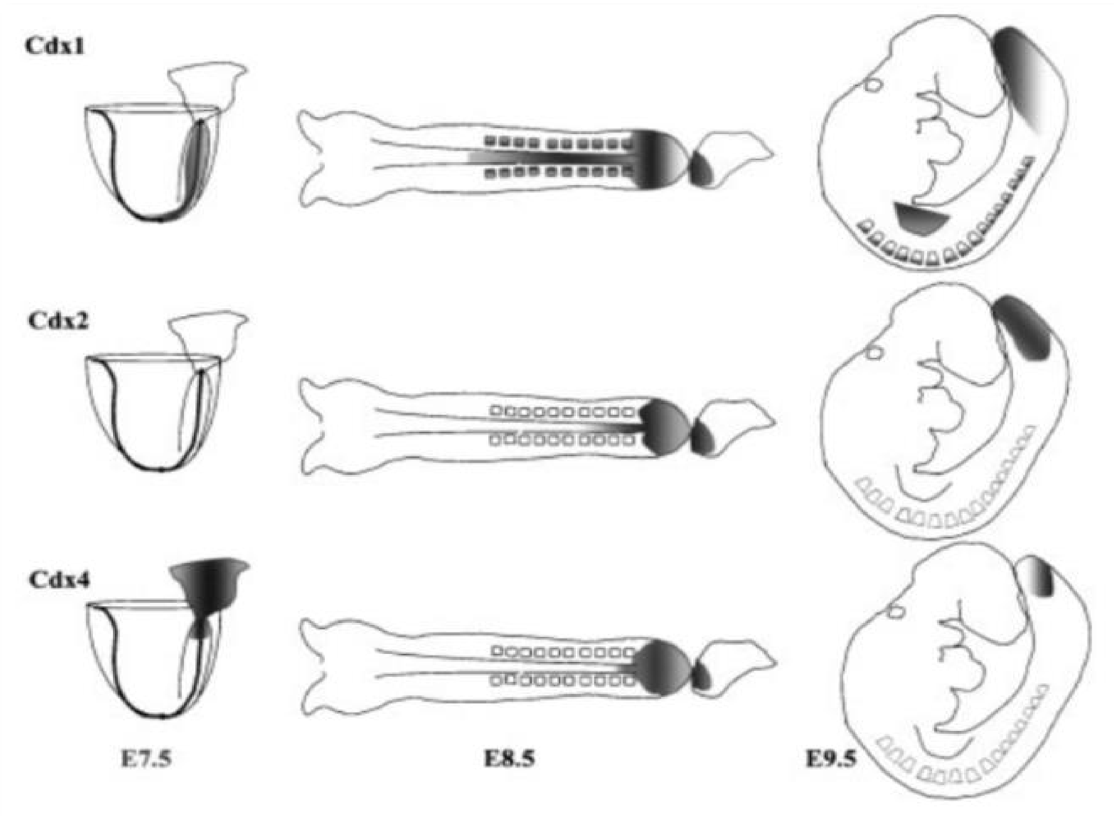


Figure 3: **Representation of *Cdx* expression in E7.5–E9.5 mouse embryos.** The left column shows transverse sections, the middle column shows lateral views of the posterior embryonic axis, and the right column shows whole-embryo lateral views. The diagrams show the progressive localization of *Cdx* gene expression along the posterior embryonic axis (Taken from Lohnes, 2003).

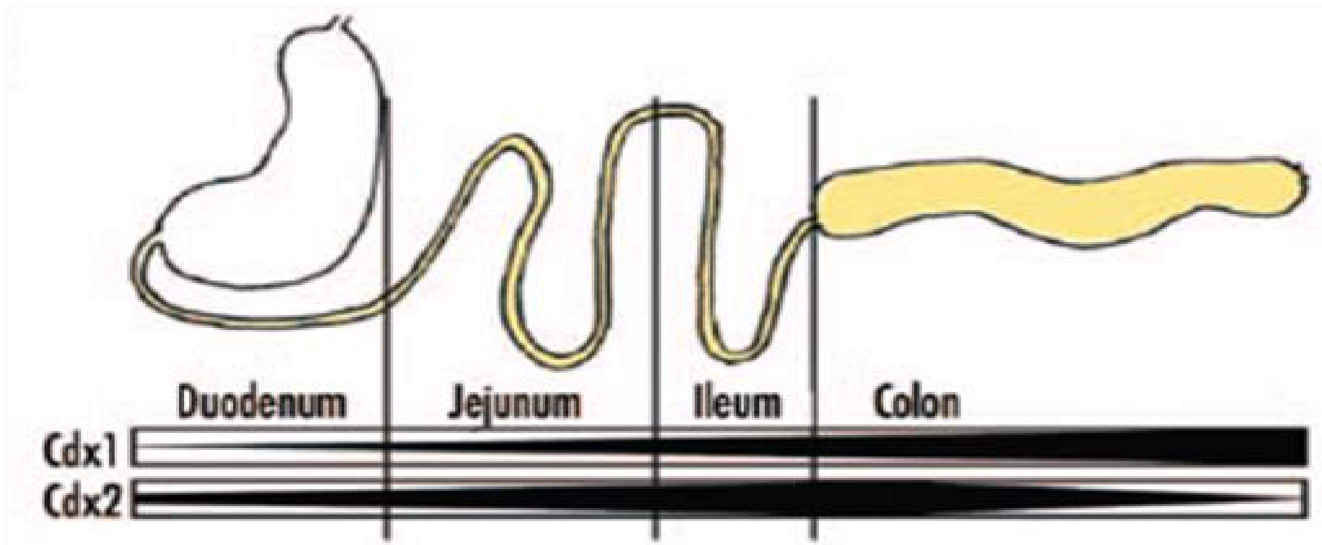


Figure 4: **Expression gradients of *Cdx1* and *Cdx2* along the intestinal tract.** The schematic illustrates the spatial distribution of *Cdx1* and *Cdx2* along the gastrointestinal tract. *Cdx2* is expressed throughout the entire length of the intestine, with higher expression levels in ileum and proximal colon, while *Cdx1* expression is progressively higher, peaking in the distal colon (Taken from Guo et al., 2004).

Gene knockouts were used to examine the critical functions of *Cdx* members. *Cdx1* null mutants have vertebral homeotic defects yet no evident intestinal phenotype (Subramanian et al., 1995). Implantation failure occurs in *Cdx2* homozygous null mutants leading to death around embryonic day (E) 3.5. On the other hand, *Cdx2* heterozygotes are viable and show vertebral homeosis and *Cdx2*-deficient gastric-like epithelium in the small intestine and colon suggesting that Cdx2 has a role in patterning of the gut (Beck et al., 2003; Chawengsaksophak et al., 1997). *Cdx4*-null mutants exhibit no significant morphological abnormalities. *Cdx4*-null mutation worsen the vertebral defects see in in *Cdx1* or *Cdx2* mutants (van Nes et al., 2006). Other compound mutants also reveal functional overlap among *Cdx* members. For instance, *Cdx1*$^{+/-}$ *Cdx2*$^{+/-}$ and *Cdx1*$^{-/-}$ *Cdx2*$^{+/-}$ compound mutants show more severe vertebral defects and tail truncation compared to those with mutations in just one of the genes (van den Akker et al., 2002). The inactivation of *Cdx4* combined with a heterozygous loss of *Cdx2*, led to loss of axial extension beyond the hindlimb placental defects and chorio-allantoic fusion failure and experienced significant growth deficiency, ultimately resulting in embryonic lethality by day 10.5 (van Nes et al., 2006). Therefore, although Cdx transcription factors are highly divergent outside of their homeodomains, they appear to functionally overlap. Consistent with this, all three members can occupy and regulate common target genes (Freund et al., 2015). This functional overlap may explain why no gut phenotype is observed in *Cdx1* null mutants.

To overcome *Cdx2* null mutant early lethality, a conditional mouse model was created by our group in which exon 2 (which encodes most of the DNA-binding homeodomain) of the *Cdx2* locus was flanked by *loxP* sites using gene targeting in embryonic stem cells (Savory et al., 2009). For intestinal-specific deletion during development, a *Cdx2*$^{f/f}$ *villin Cre-ERT* male was crossed with a *Cdx2*$^{f/f}$ female. These males express a Cre recombinase under the control of the intestine-specific *villin* promoter. The Cre protein is fused to a modified estrogen receptor ligand binding domain (ERT) that responds to tamoxifen. In the presence of this drug, Hsp90 is released from the ERT sequences, resulting in the

translocation of the Cre fusion protein to the nucleus and excision of the crucial floxed *Cdx2* sequences (Figure 5). The same process has been used to generate *Cdx1-Cdx2* conditional double mutants (DKO) by crossing a *Cdx1*$^{-/-}$ - *Cdx2*$^{f/f}$ *villin Cre-ERT* male with a *Cdx*$^{-/-}$ - *Cdx2*$^{f/f}$ female (Hryniuk et al., 2012).

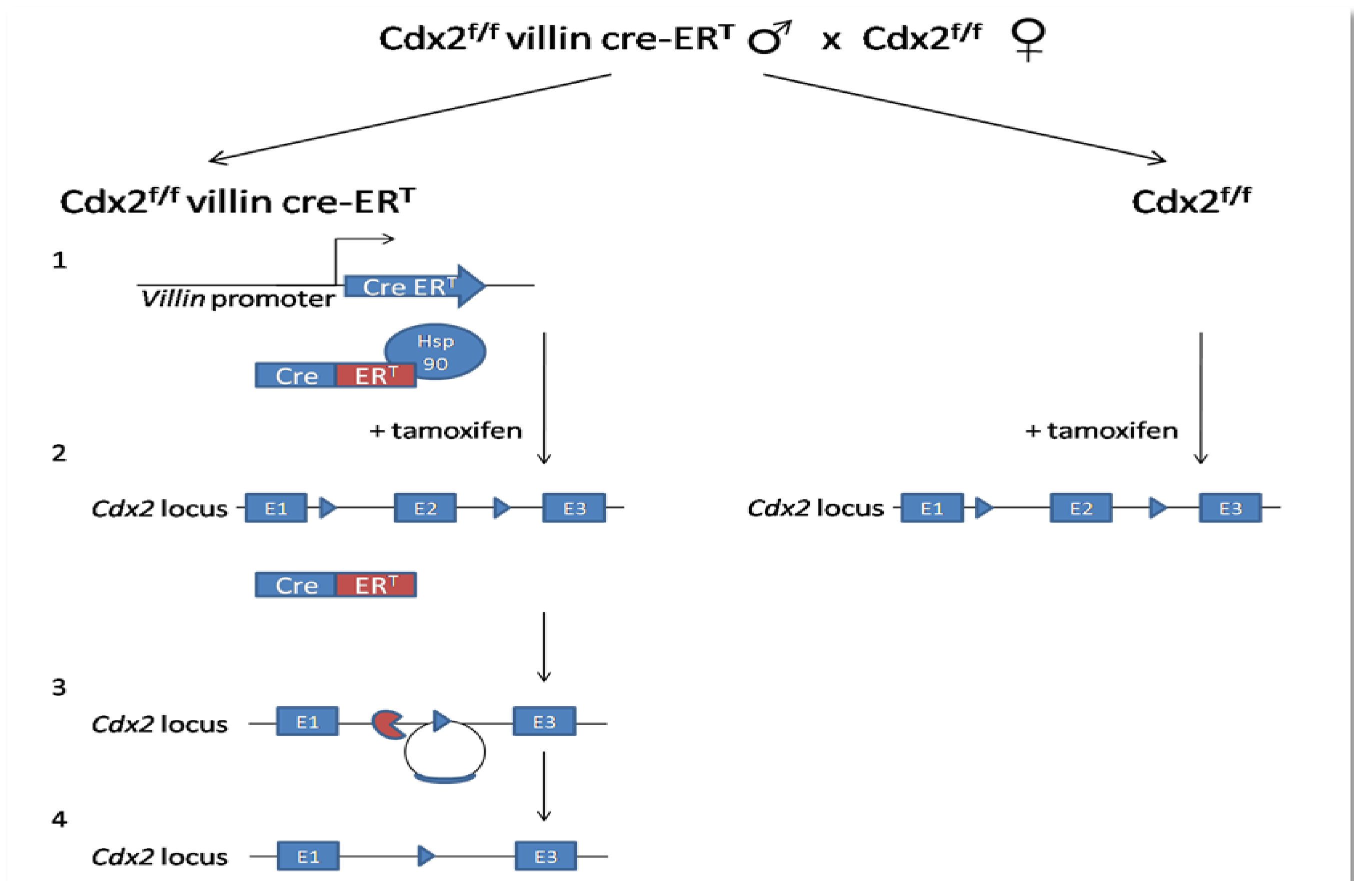


Figure 5: ***Cdx2* conditional mutagenesis strategy.** A *Cdx2*$^{f/f}$ *villin Cre-ERT* male is bred with a *Cdx2*$^{f/f}$ female. [1] *Cre-ERT* is transcribed under the control of the villin promoter and consists of a Cre protein fused to a modified estrogen receptor ligand binding domain (ERT) that only responds to tamoxifen. In the absence of tamoxifen, association with Hsp90 results in cytoplasmic sequestration of the chimeric protein. [2] Exon 2 of the *Cdx2* locus is flanked by loxP sites. For *Cdx2* conditional knockout during development, pregnant females are gavaged with tamoxifen which releases Hsp90 from the *Cre-ERT* fusion protein, resulting in the translocation of the Cre fusion protein to the nucleus. [3-4] The Cre protein recognizes the loxP sites, and a recombination event occurs, resulting in the excision of the *Cdx2* sequences. Similarly, *Cdx1*$^{-/-}$ - *Cdx2*$^{f/f}$ females can be bred with *Cdx1*$^{-/-}$ - *Cdx2*$^{f/f}$ *villin Cre-ERT* males and the same approach used to produce *Cdx1:Cdx2* double mutants (Taken from Grainger, 2012).

Through this conditional mutagenesis strategy, our lab discovered that the small intestine of *Cdx2* conditional mutant embryos exhibits stomach-like features, as suggested by the presence of H+/K+ ATPase, a key enzyme in the parietal cells of the stomach epithelium of the mutants (Grainger et al., 2010). These findings were further validated through RT-PCR analysis of stomach-specific markers (*Sox2*, *Muc1*, and *Ghrl*), which showed elevated expression levels in *Cdx2* mutants relative to controls. These results suggest that *Cdx2* deletion disrupts intestinal patterning, leading to a transformation of the small intestine into a pyloric stomach-like structure.

Our lab further investigated the role of Cdx2 in the adult gut. In this study, the deletion of *Cdx2* was induced in adult mice by administering 1 mg of tamoxifen via oral gavage, which resulted in a complete loss of Cdx2 expression. This caused severe effects, with mice dying within seven days due to impaired intestinal function. To better understand the effects of *Cdx2* deletion, the expression of markers associated with proliferation (*Ki67*), stem cells (*Olfm4*), and enterocytes, including intestinal alkaline phosphatase (*IAP*), intestinal fatty acid-binding protein (*IFABP*) and sucrase-isomaltase (*SI*) was examined. A marked reduction in the expression of all these markers in the *Cdx2* conditional mutant mice was observed. This indicates that Cdx2 plays a pivotal role in the differentiation and function of various specialized cell types critical to gut integrity and homeostasis (Hryniuk et al., 2012).

To investigate the effects of chronic *Cdx2* loss while avoiding early lethality, a mosaic knockout strategy was used. By administering a lower dose of tamoxifen (0.25 mg), conditional mutants survived similarly to control mice. Analysis of these mosaic mutants revealed areas in the small intestine lacking *Cdx2* which exhibited stomach-like characteristics and expressed gastric markers, confirming the critical role of Cdx2 in intestinal patterning in adult mice (Hryniuk et al., 2012).

Cdx2 and colon cancer

In addition to its role in intestinal differentiation and patterning, Cdx2 has also been implicated in colon tumorigenesis. In 30% of human colorectal cancer (CRC), *Cdx2* expression is lost, and this is correlated with a higher tumor grade (Baba et al., 2009; Lugli et al., 2008). More recently, it was found that *Cdx2* loss is a useful biomarker for adjuvant therapy in stage II/III colon cancer patients (Dalerba et al., 2016).

In humans, colorectal cancer (CRC) often begins with mutations in the Adenomatous Polyposis Coli (*APC*) gene. *APC* plays a crucial role in the degradation of β-catenin, a key protein in the canonical Wnt signaling pathway. Mutations in *APC* cause β-catenin to accumulate leading to uncontrolled activation of the canonical Wnt pathway, driving excessive cell growth leading to polyps, a precursor for CRC (Y. Zhang et al., 2017). Previous work showed that, in APC mutant colonic epithelial cells, reducing the expression of *Cdx2* causes additional imbalance, leading to increased cell proliferation and decreased apoptosis and supporting a role for Cdx2 in CRC (Aoki et al., 2003).

To further model the impact of *Cdx* loss on CRC, $APC^{Min/+}$ mice were crossed with either *villin Cre-ERT* $Cdx2^{f/f}$ or *villin Cre-ERT* – $Cdx1^{-/-}$ - $Cdx2^{f/f}$ mice and *Cdx2* deleted using the mosaic knockout strategy (Hryniuk et al., 2014). The resulting *Cdx-Min* compound mutants had more tumors in the small intestine compared to *Min* controls. Additionally, polyps in *Cdx2-Min* animals were spread throughout the large intestine, whereas in *APC* mice, they were limited to the proximal colon. Simultaneous deletion of *Cdx1* further increased the number of polyps, particularly in the distal colon, resembling the distribution of tumours in human CRC and showing, for the first time, a role for Cdx1 in the intestine.

Cdx2 and IBD

Prior work (Calon et al., 2007) demonstrated a link between Cdx2 and inflammation using dextran sodium sulfate (DSS)-induced colitis in $Cdx2^{+/-}$ mice. These mice exhibited a small increase in intestinal

permeability and heightened susceptibility to DSS-induced colitis, suggesting that Cdx2 plays a protective role in maintaining intestinal barrier function during DSS-induced inflammation. This is further supported by the observation that Cdx2 regulates several target genes crucial for intestinal barrier integrity, such as adhesion molecules (*LI-cadherin, E-cadherin* and *Claudin-2*) and IBD-associated genes (*HNF4α*, *Meprin 1A*, and *Mucin 2*) Hinoi et al., 2002; Sakaguchi et al., 2002). Together, these findings imply that *Cdx2* preserves intestinal homeostasis and may be a key player in IBD.

NF-kB pathway and Cdx2

Nuclear factor-κB (NF-κB) is the most extensively studied and well-characterized pathway in the inflammatory response. NF-κB is a family of transcription factors comprising five members: *RelA* (p65), *RelB*, *c-Rel*, *p50/p105* (*NF-κB1*), and *p52/p100* (*NF-κB2*) (T. Liu et al., 2017). These factors can form various homo- or heterodimers that regulate the expression of numerous genes involved in immune response and inflammation (Oeckinghaus & Ghosh, 2009). The NF-κB pathway is activated through two primary mechanisms, the canonical and non-canonical pathways, each playing a crucial role in immune and inflammatory processes.

The canonical NF-κB pathway is initiated by various signals, including activation of pattern recognition receptors (PRRs), such as Toll-like receptor 4 (TLR4) by bacterial lipopolysaccharide (LPS), members of the TNF receptor (TNFR) superfamily, and receptors involved in T-cell and B-cell signaling (H. Zhang & Sun, 2015). LPS binds to TLR4, triggering a downstream signaling cascade that activates adaptor proteins. These adaptors recruit and activate kinases, which ultimately stimulate the IKK complex. Similarly, Tumor Necrosis Factor (TNF-α) binds to TNFR, leading to the activation of the IKK complex. Once activated, IKK phosphorylates IκBα, leading to its ubiquitination and subsequent degradation. This frees NF-κB dimers, such as p50/p65, enabling their nuclear translocation and

expression of target genes, including pro-inflammatory cytokines and inflammasome components (Figure 6) (T. Liu et al., 2017).

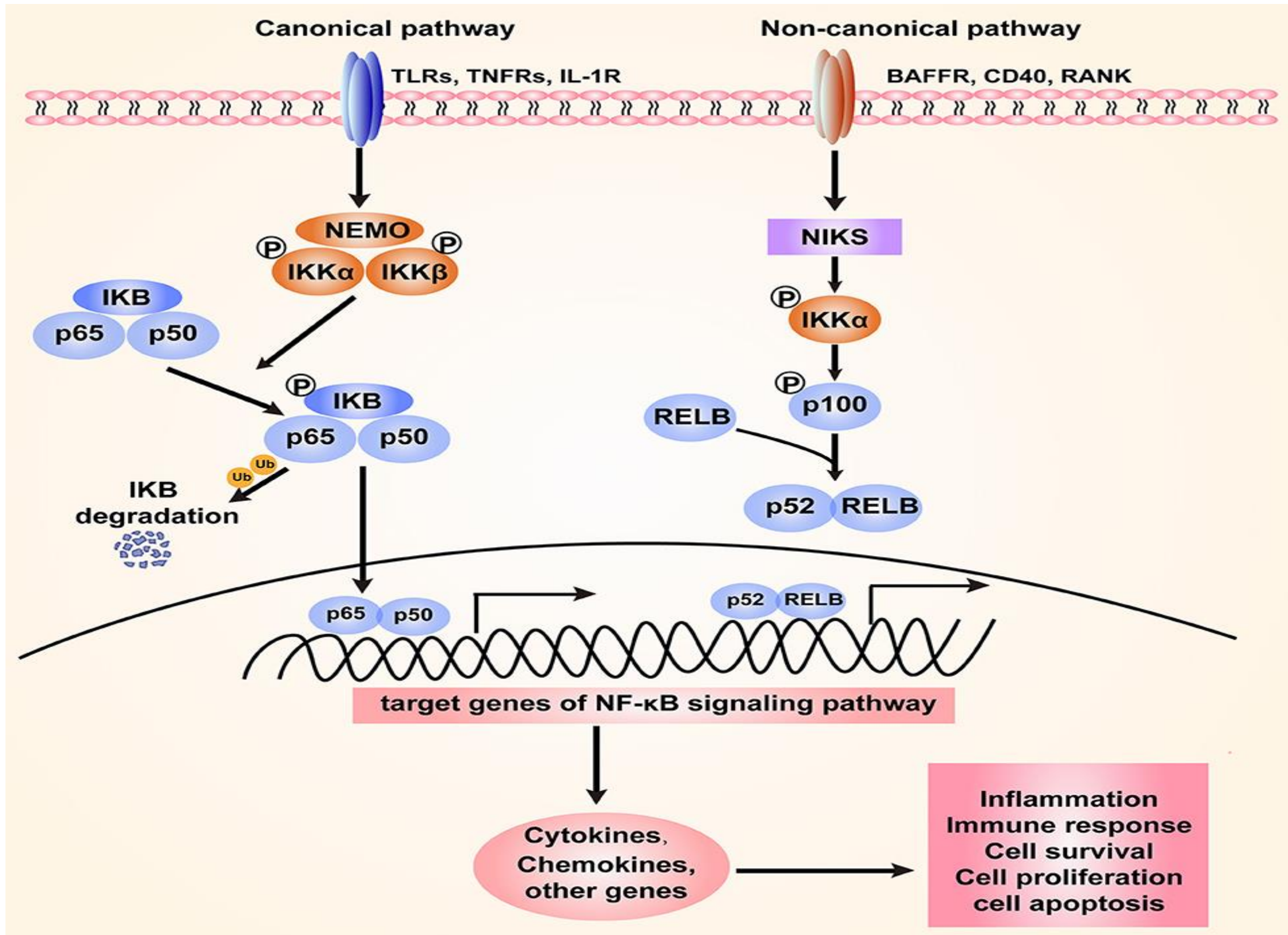


Figure 6: **Canonical and non–canonical NF-κB Signaling Pathways.** In the canonical pathway, activation is triggered by receptors such as TLRs, TNFRs, and IL-1R, leading to the phosphorylation and activation of the IKK complex (comprising NEMO, IKKα, and IKKβ). In the non-canonical pathway, activation occurs via receptors such as *BAFFR*, *CD40*, and *RANK* and involves NIK-mediated phosphorylation of IKKα, with both pathways leading to nuclear translocation of NF-κB members (Taken from Peng et al., 2020).

While NF-κB plays a pivotal role in controlling immune responses, inflammation, and cell survival, another key signaling pathway that intersects with NF-κB is the PI3K/AKT pathway. Phosphatase and tensin homolog (PTEN), a lipid phosphatase, functions as a negative regulator of the PI3K/AKT pathway by dephosphorylating PIP3, which prevents AKT activation (Georgescu, 2010). PTEN has long been recognized as a critical regulator of both embryonic development and the maintenance of tissue

homeostasis in adults (Ali et al., 1999). Through its regulation of PI3K/AKT, PTEN indirectly influences NF-κB activity, helping to maintain a balance between inflammation and cell survival (Oeckinghaus et al., 2011). When the PI3K/AKT pathway is disrupted, such as through the loss of PTEN function, the pathway becomes over activated, leading to constitutive activation of both PI3K/AKT and NF-κB. This dysregulation can promote increased cell survival and chronic inflammation.

Interestingly, there is also evidence for PTEN and NF-κB regulating Cdx2. For example, a study identified two putative NF-κB binding sites in the *Cdx2* promoter, highlighting a potential direct transcriptional regulation by NF-κB (Kim et al., 2002). Subsequent work showed that the *Cdx2* promoter can be bound by both p50/p50 homodimers and p65/p50 heterodimers, with contrasting effects: the p50/p50 homodimer increases *Cdx2* promoter activity, while the p65/p50 heterodimer decreases it (Kim et al., 2002). This dual regulation suggests that PTEN, through its suppression of PI3K/Akt, plays a role in Cdx2 expression via stimulating the DNA binding activity of the NF-κB p50/p50 homodimer and increasing *Cdx-2* expression (Figure 7).

Further work revealed that activation of the NF-κB signaling pathway by the pro-inflammatory cytokine TNF-α, a key mediator of inflammation in CD and UC, has the opposite effect on Cdx2 compared to PTEN, inhibiting its expression in the colon cancer cell line HT29 (Kim et al., 2002). This suggests that pro-inflammatory signals, such as TNF-α, which stimulate the DNA binding activity of the p65/p50 heterodimer, suppresses Cdx2, leading to the downregulation of Cdx target genes that protect the intestine. Notably, (Boyd et al., 2010) have identified several IBD susceptibility genes, including *CLDN2*, *MEP1A*, *LAMC2*, and *MUC2*, which are regulated by Cdx2 (Banerjee et al., 2009; Barrett et al., 2008; Coskun, 2014; Coskun et al., 2011, 2017; Moehle et al., 2006).

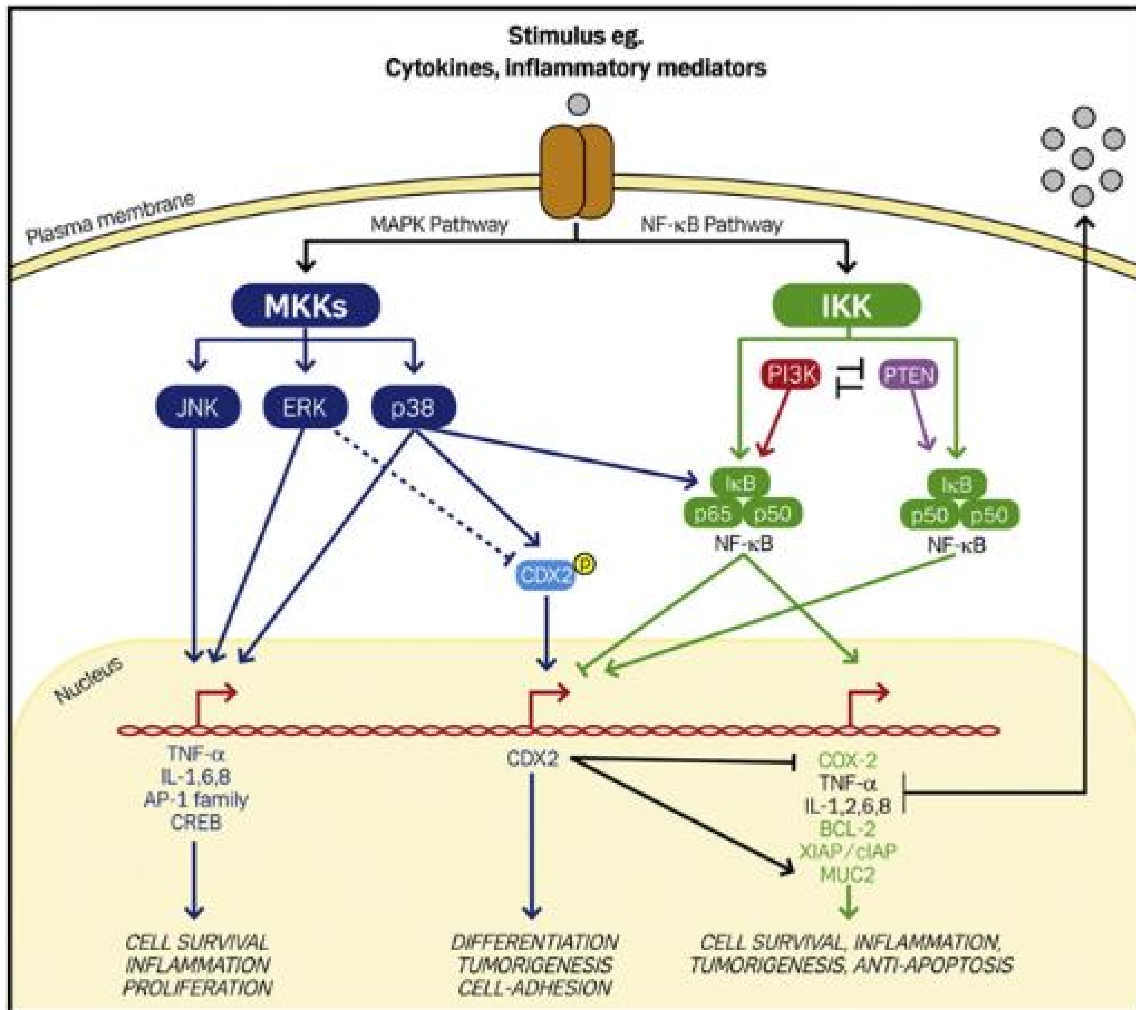


Figure 7: **Regulation of *CDX2* expression by inflammatory mediators.** External stimuli, such as cytokines, trigger the MAPK or NF-κB signaling cascades to impact *CDX2* expression. Both p38 and ERK can phosphorylate CDX2, with p38 enhancing its transcriptional activity and ERK suppressing it. *CDX2* expression is upregulated by PTEN through the NF-κB pathway, while PI3K signaling antagonizes this effect. (Taken from Coskun et al., 2011).

TNF-α may therefore contribute to intestinal barrier dysfunction and increased susceptibility to IBD via mis-regulation of Cdx target genes, highlighting the critical role of Cdx2 in intestinal health and its potential importance in IBD pathogenesis.

Research conducted in our lab (Jahan et al., 2022) has provided additional insight into the role played by Cdx, particularly within the colon epithelium. This study revealed an upregulation of 19 pro-inflammatory genes in *Cdx* conditional mutant colon, including pivotal cytokines and chemokines such as TNFα, IL-1β, IL-18, CXCL1, and CCL2. These cytokines and chemokines are hallmark indicators of an activated NF-κB signaling pathway, consistent with Cdx function playing a protective role against inflammation in the intestine. Furthermore, our findings suggest that *Cdx2* loss may operate, at least in part, through the activation of the NLRP3 inflammasome, as described below.

The innate immune system and inflammasomes

Inflammasomes are multi-protein complexes found in cells, particularly in immune cells like macrophages, where they play a critical role in the body's innate immune response. Acting as sentinels for cell damage and infection, inflammasomes trigger inflammation to help defend the body against potential threats. These complexes are typically composed of three core components: the sensor, the adaptor, and the effector proteins. Together, these components enable the inflammasome to detect danger, assemble into an active complex, and initiate an immune response (Dai et al., 2023). At the forefront of this process are the Pattern Recognition Receptors (PRRs), which serve as the body's first line of defense. PRRs recognize specific molecular patterns, known as Pathogen-Associated Molecular Patterns (PAMPs), which are associated with pathogens, and Damage-Associated Molecular Patterns (DAMPs), which are released from damaged or stressed cells. There are several types of PRRs, including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), AIM2-like receptors (ALRs), NOD-like receptors (NLRs), and the cGAS/STING pathway (Wicherska-Pawłowska et al., 2021).

Inflammasome-forming NOD-like receptors

Among the family of PRRs, a particularly important group is the NOD-like receptors (NLRs) (Davis et al., 2014). The NLR family comprises several members, including NLRP1, NLRP3, NLRP6, NLRC4,

and NLRP12, each of which responds to distinct stimuli. These receptors are defined by multiple functional domains. The N-terminal domain is vital for regulating protein-protein interactions and controlling the inflammasome activation process. The central NACHT domain contains an NTP-binding site, and the hydrolysis of ATP is essential for inducing the conformational changes required for NLR oligomerization, a critical step in inflammasome complex assembly. Additionally, the LRR (leucine-rich repeat) domain plays a key role in detecting danger signals, such as PAMPs and DAMPs, triggering the activation of the inflammasome (Almeida-da-Silva et al., 2023).

Upon detecting danger signals, NLRs undergo conformational changes that lead to the assembly of inflammasomes. This process involves the recruitment of the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), which interacts with NLRs through its pyrin domain (PYD) and forms visible speck-like structures. Through its CARD (Caspase Activation and Recruitment) domain, ASC then recruits pro-caspase-1, which undergoes autocatalytic cleavage to produce active caspase-1 which then processes pro-IL-1β and pro-IL-18, resulting in the maturation and release of IL-1β and IL-18, as well as cleavage of gasdermin D (Davis et al., 2014).

The NLRP3 inflammasome

The NLRP3 inflammasome has gained significant attention due to its involvement in numerous inflammatory diseases such as Cryopyrin-associated periodic syndrome (CAPS), IBD, Rheumatoid Arthritis (RA), and several neurodegenerative diseases (Y. Chen et al., 2023). The NLRP3 inflammasome is a complex composed of NLRP3, ASC, and Pro-caspase-1 (Tourkochristou et al., 2019). Like most NLRs which recruit ASC and activate caspase-1, NLRP3 also plays a critical role in caspase-1 maturation, as well as the cleavage and release of mature IL-1β and IL-18, both of which are pivotal mediators of inflammation.

Inflammasome activation requires strict regulation to prevent excessive or uncontrolled immune responses. Typically, it follows a two-step mechanism: an initial 'priming' phase that prepares the inflammasome components and a subsequent 'activation' phase that triggers the inflammatory cascade (Swanson et al., 2019). The priming phase serves to enhance the expression of NLRP3 inflammasome components, including NLRP3 itself as well as caspase-1, pro-IL-1β/18 and ASC. This process is triggered by the recognition of PAMPs or DAMPs, which interact with pattern recognition receptors PRRs such as TLRs or NOD2, or cytokines such as TNF and IL-1β which promote NF-κB activation, leading to increased gene expression of inflammasome elements (Bauernfeind et al., 2009; Franchi et al., 2009) (Figure 8). Another key function of the priming phase is to initiate post-translational modifications (PTMs) of the NLRP3 protein, which are crucial for controlling its activity and ensuring the inflammasome does not overreact (Swanson et al., 2019).

The second "activation" phase, triggers the assembly and activation of the inflammasome through mechanisms that are not fully understood. Several factors can serve as triggers, including metabolic changes such as ATP depletion. These triggers can lead to the loss of potassium (K+) from cells, which is a critical step for inflammasome activation. ROS, produced during oxidative stress, also play an essential role in activating the NLRP3 inflammasome by disrupting cellular redox balance (Muñoz-Planillo et al., 2013; Pétrilli et al., 2007; Swanson et al., 2019).

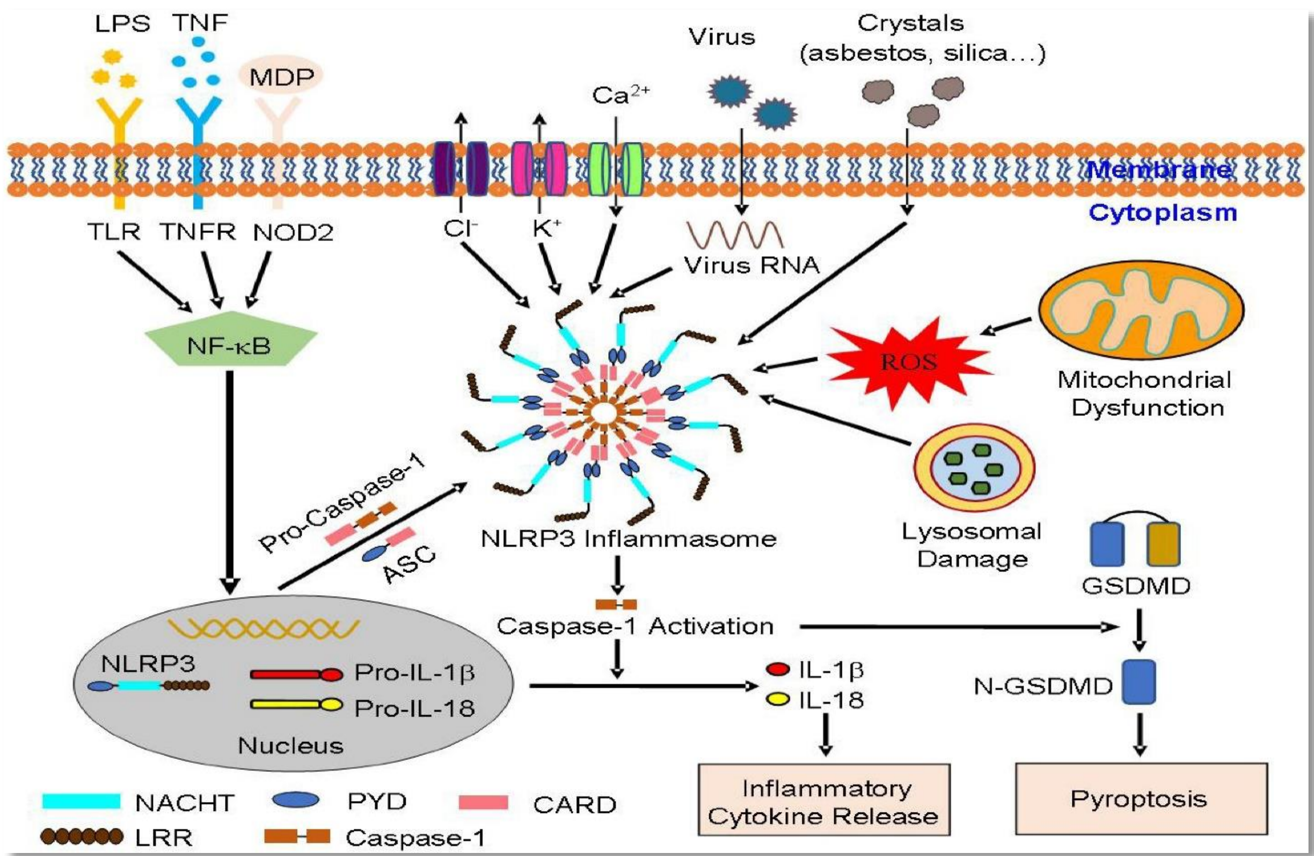


Figure 8: **The structure and activation of NLRP3 inflammasome.** The NLRP3 inflammasome consists of NLRP3, ASC, and pro-caspase 1. A two-step mechanism leads to NLRP3 inflammasome activation, including the priming and the activation step; in the process of NLRP3 inflammasome activation, activated caspase-1 transforms pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18, resulting in the release of inflammatory cytokines (Taken from Shi et al., 2020).

Changes in ion concentrations, especially the efflux of potassium K+ from the cell and increased calcium (Ca2+) levels inside the cell, are recognized as early signals for inflammasome activation (Murakami et al., 2012; Swanson et al., 2019; Triantafilou et al., 2013; Yaron et al., 2015). Mitochondrial dysfunction also plays a role, with the release of mitochondrial DNA being detected by immune receptors, signaling stress and promoting inflammasome assembly (Swanson et al., 2019; Zhong et al., 2018).

Environmental factors, such as exposure to toxins like nickel and asbestos, as well as crystalline substances (e.g., cholesterol, uric acid crystals, and alum), can also activate the NLRP3 inflammasome. This suggests the inflammasome's involvement in reaction to environmental pollutants, which could contribute to various inflammatory diseases (De Nardo & Latz, 2011; Swanson et al., 2019).

Post-translational regulation of the NLRP3 inflammasome

Proper regulation of the NLRP3 inflammasome is crucial to prevent an overly aggressive immune response and excessive inflammation. Various post-translational modifications (PTMs, including phosphorylation, sumoylation and ubiquitination, work together to regulate NLRP3 activation, ensuring it responds accurately to specific stimuli while preventing the excessive immune activity that could lead to the development of autoimmune diseases, inflammatory bowel disease (IBD), or other chronic inflammatory conditions (Swanson et al., 2019).

Phosphorylation of specific residues in NLRP3 plays a critical role in modulating its stability and the assembly of the inflammasome. Notably, phosphorylation of Ser5 within the PYD disrupts NLRP3's interaction with ASC (N. Song et al., 2017). This modification is mediated by the c-Jun N-terminal kinase 1 (*JNK1*), which activates in response to cellular stress signals, thereby regulating NLRP3 inflammasome activation and function.

Sumoylation involves the covalent attachment of SUMO (Small Ubiquitin-like Modifier) to target proteins, a process that is mediated by a highly conserved enzymatic cascade. This cascade consists of an E1 activating enzyme, an E2 conjugating enzyme (such as UBC9), and an E3 ligase, which provides substrate specificity. The E3 ligase MAPL/MUL1 mediates sumoylation of NLRP3 in resting cells, keeping it inactive. During activation, desumoylation enzymes, specifically SENP6 and SENP7, remove SUMO modifications from NLRP3, enabling the assembly of the inflammasome complex.

Dysregulation of sumoylation or desumoylation can lead to uncontrolled NLRP3 activation, contributing to inflammatory disorders like Cryopyrin-Associated Periodic Syndromes (CAPS) (Barry et al., 2018).

Ubiquitination also regulates NLRP3 through a multi-step enzymatic process driven by the sequential actions of E1 activating enzymes, E2 conjugating enzymes, and E3 ligases. E1 enzymes activate ubiquitin in an ATP-dependent manner, transferring it to E2 enzymes, which then work in concert with E3 ligases to ubiquitinate specific lysine residues on target proteins. In resting macrophages, NLRP3 is targeted for proteasomal degradation by the FBXL2 E3 ligase complex. Following LPS priming, this process is inhibited, increasing NLRP3 stability (Han et al., 2015). Other E3 ligases, like TRIM31, also regulate NLRP3 through Lys48-linked ubiquitination, promoting its degradation under inhibitory conditions. This dynamic balance between ubiquitination and deubiquitination ensures tight regulation of NLRP3 activity to prevent aberrant inflammasome activation. (Song et al., 2016).

A novel Cdx-TRIM31-NLRP3 pathway

Work from our lab (Jahan et al., 2022) found that *Cdx* conditional mutant intestine exhibited elevated levels of NLRP3, IL-1β, and caspase-1, while *TRIM31* expression decreased. Further investigation revealed the presence of a functional Cdx-Responsive Element (CDRE) in the promoter region of the *TRIM31* gene. Chromatin Immunoprecipitation (ChIP) analysis confirmed that Cdx2 directly binds to the *TRIM31* locus, establishing a transcriptional regulatory mechanism for *TRIM31* in response to Cdx2. Additionally, treating these mutants with the NLRP3 antagonist CY-09 reduced pro-inflammatory cytokine levels and extended their survival, supporting the existence of a Cdx2-TRIM31-NLRP3 pathway that regulates intestinal inflammation and cytokine release. Similar patterns of reduced *CDX2* and *TRIM31* levels, along with increased IL-1β and TNF-α, were observed in UC patients, suggesting that this pathway is conserved and may play a role in IBD.

NLRP3 inflammasome and pyroptosis

Activation of the NLRP3 inflammasome triggers not only the release of inflammatory cytokines but also pyroptosis, an inflammatory form of programmed cell death. This process is driven largely by cleavage of Gasdermin D (GSDMD), one of the most studied pyroptosis-mediating proteins (J. Shi et al., 2015). GSDMD is comprised of two main domains: the N-terminal (NT-GSDMD; also known as p30), which is responsible for pore formation in the plasma membrane, and the C-terminal (CT-GSDMD; also called p20), which inhibits pore formation by binding to NT-GSDMD (in the context of the full-length protein) and keeping the protein in an auto-inhibited state (Dai et al., 2023; Devant & Kagan, 2023).

Several regulatory pathways govern GSDMD-mediated pyroptosis, broadly classified into canonical, non-canonical, and alternative pathways (Dai et al., 2023) (Figure 9). The canonical pathway is triggered by the activation of inflammasomes including NLRP3, AIM2, or NLRC4, leading to the activation of caspase-1. Caspase-1 then cleaves GSDMD, releasing the NT-GSDMD fragment, which forms pores in the plasma membrane, causing pyroptosis (Broz & Dixit, 2016).

In the non-canonical pathway, caspase-4 and caspase-5 (in humans) or caspase-11 (in mice) are activated upon interaction with lipopolysaccharides (LPS) from Gram-negative bacteria. These caspases cleave GSDMD to induce pyroptosis independently of canonical inflammasome signaling (Kayagaki et al., 2015).

Alternative pathways include mechanisms such as caspase-8-driven pyroptosis, observed under specific infectious or apoptotic conditions, and caspase-3-mediated activation of Gasdermin E (GSDME).

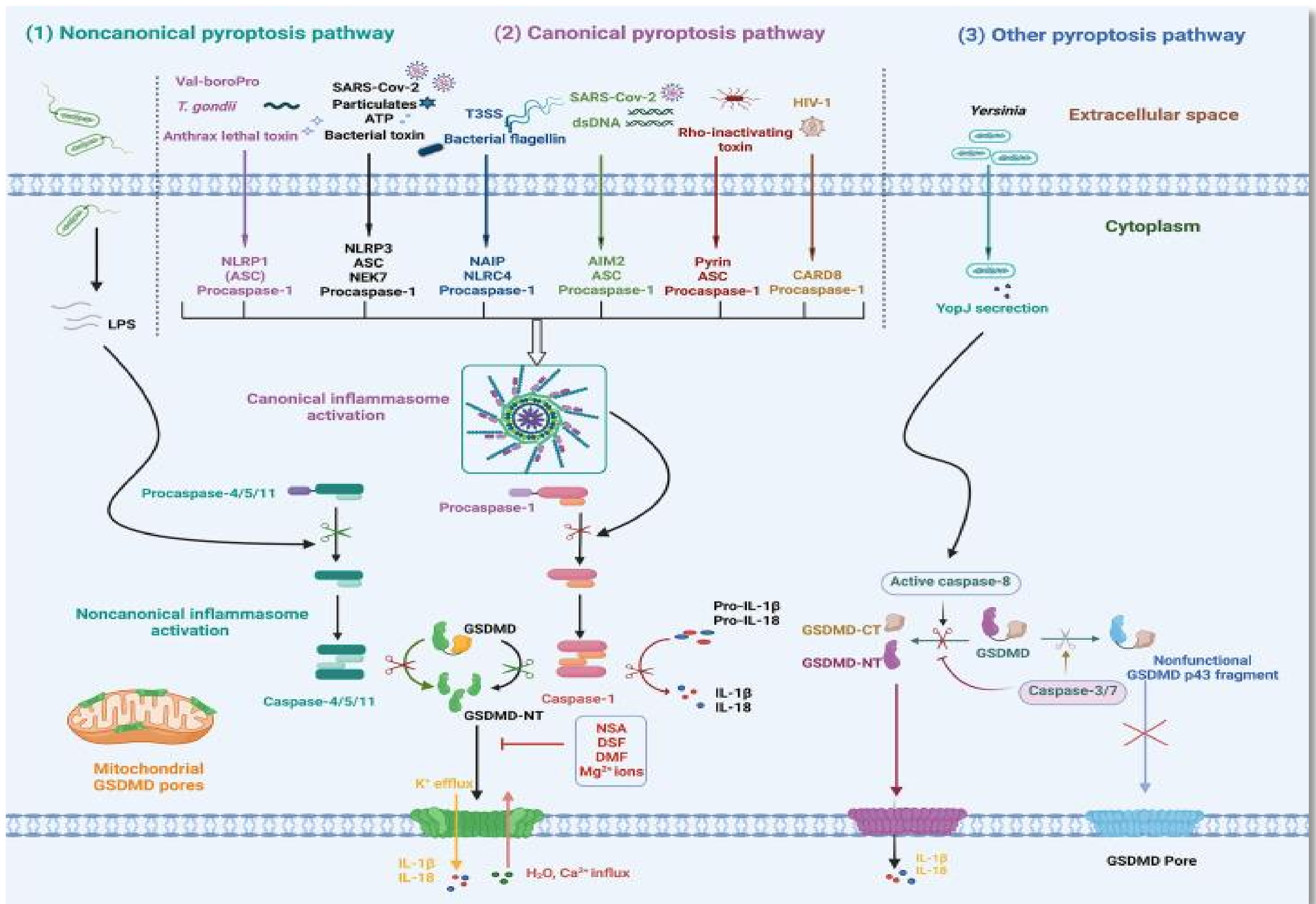


Figure 9: **Regulation of Gasdermin D-mediated pyroptosis.** In reaction to different PAMPs or DAMPs, inflammatory caspases-1/4/5/11 are activated, triggering the cleavage of GSDMD. Furthermore, during Yersinia infection, caspase-8 activation causes the cleavage of GSDMD into NT-GSDMD. GSDMD-NT then oligomerizes and integrates into the plasma membrane, creating pores that induce ion fluxes, the release of inflammatory cytokines, and pyroptosis (Taken from Dai et al., 2023).

Cleavage of GSDME by caspase-3 generates an N-terminal fragment (NT-GSDME) that functions similarly to NT-GSDMD in forming membrane pores, though it is activated by apoptotic caspases rather than inflammatory caspases. GSDME cleavage results in pyroptosis-like cell death, often occurring in cancer therapies involving chemotherapeutic agents (Sarhan et al., 2018; Wang et al., 2017).

In addition to activating pyroptosis, caspase-3/7 can also cleave GSDMD to form an inactive fragment, serving as a feedback mechanism to prevent excessive tissue damage. Conversely, caspase-8 can cleave

both GSDMD and GSDME, linking apoptotic and pyroptotic pathways under certain conditions, such as Yersinia infection or TAK1 inhibition (Dai et al., 2023; Sarhan et al., 2018). These diverse mechanisms underscore the complex interplay between cell death pathways, where pyroptotic stimuli can activate apoptosis, and apoptotic stimuli can modulate pyroptosis.

While pyroptosis can serve as a critical immune defense mechanism by eliminating infected cells and promoting inflammation, its excessive activation can contribute to pathological tissue damage, as seen in inflammatory diseases and cancer treatments. The dual regulation of Gasdermin proteins by caspases highlights the versatility of pyroptosis as both a host defense and a potential driver of disease progression, depending on the context.

Ultimately, these pathways converge largely on a common outcome: the cleavage of GSDMD leading to the release of its NT-GSDMD fragment. NT-GSDMD, which has strong lipid-binding properties, interacts with phospholipids like phosphatidylinositol and phosphatidylserine that are enriched in the inner leaflet of the plasma membrane (Dai et al., 2023). Guided by electrostatic and hydrophobic interactions, NT-GSDMD then oligomerizes to form large, stable, pores in the membrane, typically 10–20 nm in diameter (Aglietti et al., 2016; Liu et al., 2016). This pore formation disrupts cellular ionic homeostasis, allowing the efflux of potassium and the influx of water, ultimately causing cell lysis. At the same time, the pores serve as conduits for the release of the pro-inflammatory cytokines IL-1β and IL-18, amplifying the inflammatory response (Evavold et al., 2018). Thus, while the triggering pathways may differ, the execution phase of pyroptosis universally involves NT-GSDMD-mediated pore formation as its defining event.

GSDMD can be regulated by means in addition to its cleavage. One well-known posttranslational modification (PTM) of GSDMD is the oxidation of cysteine at residue Cys192, which is triggered by

reactive oxygen species (ROS) often produced during inflammasome activation. This oxidation boosts NT-GSDMD's ability to move to the plasma membrane (Devant & Kagan, 2023; Evavold et al., 2018). On the other hand, GSDMD activity can be inhibited by fumarate, which modifies Cys192 through succination. This prevents GSDMD from interacting with caspase-1, blocking pyroptosis and serving as an anti-inflammatory mechanism (Humphries et al., 2020).

While most studies on GSDMD have focused on immune cells, its role in non-immune cells is gaining attention. Research highlighted GSDMD's critical role in the intestinal epithelium, particularly in regulating mucus secretion from goblet cells to uphold the intestinal mucosal barrier (Zhang et al., 2022). Further, a recent study demonstrated that GSDMD protects intestinal epithelial cells against bacterial infections through its N-terminal fragment (Li et al., 2024). Dysregulated pyroptosis due to abnormal activation of GSDMD has also been associated with a variety of diseases, including autoimmune disorders, cancer, and neurodegenerative conditions (Dai et al., 2023).

Given that GSDMD is involved in regulating inflammation and immune responses, additional research on this protein could help identify regulatory mechanisms which could identify novel therapeutic strategies aimed at targeting its cleavage and pore formation to treat inflammatory diseases. Furthermore, GSDMD's role in different cell types, such as in intestinal epithelial cells, requires further exploration to better understand its diverse functions.

Hypothesis

As loss of Cdx function induces NLRP3 activity in intestinal epithelial cells, I hypothesize that this impacts intestinal inflammation through GSDMD cleavage leading to enhanced pyroptotic cell death.

Objectives

Objective 1: Compare GSDMD processing between control and *Cdx* mutant mouse intestinal epithelial cells.

Objective 2: Assess pyroptosis in *Cdx* mutant epithelial cells.

Objective 3: Determine if NT-GSDMD translocates from the cytoplasm to the membrane in *Cdx* mutant cells.

Chapter 2

Materials and Methods

Mouse models and treatments

Cdx2$^{f/f}$, *Cdx1*$^{-/-}$, and *Villin-Cre ER*^{T} mice have been previously described (el Marjou et al., 2004; Savory et al., 2009; Subramanian et al., 1995). To derive mice lacking *Cdx* in the intestine, *Cdx1*$^{-/-}$*Cdx2*$^{f/f}$ - *Villin-Cre ER*^{T} were administered 250 µL of tamoxifen (2 mg total) (Sigma; T5648-5G) in corn oil via oral gavage. The treatment will generate a *Cdx* double knockout, where both *Cdx1* and *Cdx2* are knocked out. Throughout my thesis, this condition will be referred to as *Cdx* mutant or DKO (double knockout) for consistency. *Cdx1*$^{-/-}$*Cdx2*$^{f/f}$ mice were administered corn oil alone and used as controls. After 4 days, mice were euthanized by cervical dislocation and the intestines were collected. All animal work described in this study was approved by the Animal Care and Veterinary Service of the University of Ottawa in accordance with the guidelines of the Canadian Council for Animal Care.

DNA extraction and Genotyping

Ear biopsies were collected and Extraction Master Mix (40 µL of Extraction Solution and 10 µL of TP solution Millipore Sigma; T3073) was added, ensuring the snip was fully submerged. The samples were incubated for 30 minutes at room temperature, followed by 95°C for 3 minutes. 40 µL of Neutralization Solution (Millipore Sigma; N3910) was added to each sample, vortexed for 15 seconds, and centrifuged at 6000 rpm for 3 minutes and the supernatant transferred to a fresh tube for PCR. A PCR reaction mix was prepared for each sample, consisting of 2.5 µL of genomic DNA, 12.5 µL GoTaq polymerase (Fisher Scientific; PR-M7123), 1 µL forward and reverse primers (Table 1), and 8 µL of nuclease-free water for a 25 µL total volume. The tubes were sealed with strip lids, briefly centrifuged at 1000 rpm and PCR performed using a DNA Engine Tetrad 2 (Bio-Rad). For the *Cdx2* floxed/floxed (*Cdx2*$^{f/f}$)

allele, samples were incubated at 98°C for 2 minutes, followed by 35 cycles at 96°C for 30 seconds, 57°C for 20 seconds, and 72°C for 45 seconds. For *Villin-Cre*, samples were incubated at 98°C for 3 minutes, followed by 39 cycles at 96°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1 minute and 30 seconds. Negative control reactions were was performed without DNA, while positive controls containing the allele interest were included to verify the efficiency of the PCR conditions.

To analyze samples, 20 µL of each reaction was loaded onto a 1.5% agarose gel in TAE buffer containing 20 µL of ethidium bromide. A GeneRuler 1 kb DNA ladder (ThermoFisher; SM0311) was included for size estimation. The gel was run at 120 V for 1 hour and visualized on a Bio-Rad Molecular Imager Gel Doc XR+ Imaging System.

Gene	Primer Sequence (5’ → 3’)	Product Size (bp)
Villin Cre	Forward: 5’- CAAGCCTGGCTCGACGGCC -3’	~800 bp
	Reverse: 5’- CGCGAACATCTTCAGGTTCT -3’	
Cdx2 F/F	Forward: 5’-TCTGATCCCTCCTGTATCTTCCCAGG -3’	WT band: 250 bp
	Reverse: 5’- GCTATTCAACCCGGTTAAGTCGGGG -3’	Floxed band: 30 bp

Table 1: Genotyping Primer Sequences and Product Sizes.

Intestinal cell isolation

Epithelial cells were isolated as previously described (Ge et al., 2023). Briefly, intestines from control or *Cdx* mutant (*Cdx*$^{-/-}$) mice were collected, rinsed with ice cold PBS, dissected longitudinally and cut into 2 to 3 cm pieces. These fragments were incubated in 30 mM EDTA in PBS on ice for 30 minutes, with gentle shaking every 7 minutes. After 30 minutes, the tube containing the tissue fragments was shaken vigorously for 2 minutes. The fragments were then discarded, and the cells were centrifuged at 500 x g for 5 minutes at 4°C. The resulting cell pellet was washed with cold PBS, centrifuged again at 500 x g, and gently resuspended in 3.5 mL of TrypLE Express enzyme (ThermoFisher; 12604-021) for 10 minutes at room temperature with pipetting up and down every 3-4 minutes to dissociate cell

aggregates. The cells were then filtered through a 70 µm cell strainer (Fisher scientific; 22-363-548), and 10 mL of cold PBS added to wash the strainer. After centrifuging again at 500 x g, the cells were resuspended in PBS containing DNase I at a concentration of 50 units/mL (Stemcell Technologies; 07900) and incubated for 15 minutes at room temperature. To assess cell viability, 10 µL of cells was mixed with 10 µL of Trypan Blue Stain (Gibco; 15250-061) using a counting chamber slide (ThermoFisher; C10283) and analyzed using the Countess II FL. Cell viability was typically determined to range between 85% and 95%.

Fluorescence-activated cell sorting (FACS)

Approximately 10^7 cells, isolated as described above, were resuspended in 300 µL FACS buffer + 4 mM EDTA (ThermoFisher; 00-4222-57) and incubated with 0.25 µg fluorophore-conjugated antibodies, including PE-Cy7 CD326 (EpCAM) to identify epithelial cells and PE-CD45 to identify immune cells (Table 2), for 30 minutes at 4°C. After incubation, cells were centrifuged at 500 x g for 5 mins, washed with FACS buffer + 4 mM EDTA and centrifuged again. The cell pellet was resuspended in 500 µL FACS buffer + 4 mM EDTA and 15 µL of 7-AAD dye (Table 2) was added for dead cell exclusion and incubated for 15 minutes on ice in the dark. Cells were then sorted on a Beckman Coulter MoFlo XDP. Intestinal cells were introduced into the sorter through a sample line and drop delay calibration was performed prior to sorting to optimize the sorting accuracy. Sorting was set to enrich for EpCAM-positive CD45-negative epithelial cells based on fluorescence intensity in the PE-Cy7 channel for far-red fluorescence (CD326-EpCAM) and the PE channel for red fluorescence (CD45). A live/dead cell gate was applied using 7-AAD, doublet exclusion was achieved using forward scatter (FSC) and debris excluded using side scatter (SSC) gating. Fluorescence gates were adjusted based on a negative control and single-stained controls prior to sorting.

Cells were recovered in a 15 mL tube containing Dulbecco's Modified Eagle Medium (DMEM) (Gibco; 11965-092) and 10% Fetal Bovine Serum (Multicell; 080150) on ice. The purity of post-sorted cells was assessed by running an aliquot through the MoFlo to confirm that the sorted population exhibited the expected fluorescence profile. The purified epithelial cells were then centrifuged at 500 x g for 5 mins at 4°C and prepared for Western Blot analysis.

Subcellular fractionation

Intestinal cells from WT or *Cdx*$^{-/-}$ mice were isolated using the TrypLE protocol and resuspended in fractionation buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA) supplemented with protease inhibitor cocktail (Cell Signaling; 5861S) and 1 mM DTT. The cells were incubated on ice for 15 minutes, then lysed by passage through a 26-gauge needle 10 times, followed by an additional 20 minute incubation on ice. The lysate was centrifuged at 10,000 x g for 5 minutes at 4°C, and the supernatant (containing the cytoplasmic and membrane fractions) was further centrifuged at 100,000 x g for 1 hour at 4°C. The supernatant containing the cytoplasm was collected and the pellet was washed with fractionation buffer, re-centrifuged at the same speed for 45 minutes at 4°C, and resuspended in 100 µL Laemmli buffer for Western Blot analysis.

Intestinal explants

Intestines from control and *Cdx* mutant mice were harvested and rinsed thoroughly with cold PBS. Each intestine was longitudinally opened to expose the epithelial surface and then cut into small fragments of approximately 1–2 cm in length. The tissue fragments were placed in 6-well plates (Corning; 29442-036) containing DMEM and incubated at 37°C with 5% CO_2 for 2 hours. Following incubation, the culture media was collected and centrifuged at 500 × g for 5 minutes at 4°C. The resulting supernatant was collected and analyzed by Western blot using a caspase-1 antibody (Table 2).

Western Blot

Purified epithelial cells or unfractionated intestinal cells were resuspended in RIPA lysis buffer (150 mM Sodium Chloride, 50 mM Tris-HCl, 1% Nonidet P-40, 0.5% Sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail and 10 mM Sodium Fluoride (Fisher chemical; S299-100), or lysed directly with Laemmli buffer (20% SDS, 1M Tris buffer (pH= 6.8), bromophenol blue, β-mercaptoethanol, and glycerol) containing 4 M urea. For RIPA isolation, cells were agitated for 20 mins at 4°C and then centrifuged at 21,000 x g for 20 mins at 4°C. The supernatant was collected and the protein concentration was determined using a protein assay kit (Biorad; 5000113-14-15) as per the manufacturer's instructions and analyzed on a BioTek Synergy H1 Hybrid Reader. 20µg of protein from each sample was denatured by heating at 95°C for 5 minutes in Laemmli buffer and separated by electrophoresis on a 12% SDS-PAGE (12% Acrylamide, in Tris, 10% SDS) at 180 V for 40-50 mins in running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS). Proteins were then transferred to a PVDF membrane at 100 V for 1 hour on ice in transfer buffer (192 mM Glycine, 25 mM Tris base, 10% methanol). Following transfer, the membrane was blocked for 1 hour at room temperature with 5% non-fat dry milk (BioShop; SKI400.1) in TBST (TBS + 0.1% Tween-20 (Amresco; 0777-1L)), washed 3 times with TBST for 5 minutes each and incubated overnight at 4°C with primary antibodies (Table 2) in 5% BSA (BioShop; ALB001.100) in TBST. The following day, the membrane was washed with TBST 3 times and an HRP-conjugated secondary antibody (Table 2) in 5% non-fat dry milk in TBST was added and incubated for 1 hour at room temperature. After washing 3 times for 10 minutes with TBST, the membrane was treated with Immobilon Forte Western HRP substrate (Millipore Sigma; WBLUF0500) for a few minutes and analyzed on a Biorad ChemiDoc MP Imaging System.

To assess protein loading, Western blot membranes were stripped using a Re-Blot Plus Strong Solution (Millipore Sigma; 4080245) for 20 minutes at room temperature. Following stripping, membranes were blocked with 5% milk in TBST three times for 10 minutes each. After blocking, membranes were

washed with TBST and subsequently incubated with primary antibodies against β-actin or Na^+/K^+ ATPase as loading controls.

Data was analyzed and plotted using GraphPad Prism 10. Statistical significance was assessed using an unpaired two-tailed *t*-test within the software. Error bars, representing the standard deviation (SD) of the mean, were also calculated and added using GraphPad Prism 10.

For quantification, band intensities were manually selected and analyzed using Image Lab software. Signal intensity was measured for both the protein of interest and β-actin, which was used for normalization. WT samples were set to 1 to facilitate fold change calculations. A minimum of three biological replicates, each representing an independent mouse, were analyzed.

To analyze low molecular weight proteins such as cleaved caspase-1, a Tricine gel system (38% glycerol, ddH2O, 30% Acrylamide, 3 M Tris-HCl (pH= 8.5), 1 M Tris-HCl (pH= 6.8), 10% SDS)) was used, and the gel run at 120V for 50 to 60 minutes in a Tricine gel running buffer (0.1 M Tris, 0.1 M Tricine and 0.1% SDS). All subsequent steps, were performed in the same manner as for the Western blot analysis described above.

Murine Colon Organoids

Colon organoids were generated from *Cdx1*$^{-/-}$ *Cdx2*$^{f/f}$ *-Villin-Cre ER*^{T} mice by standard means and cultured in 24-well plates (Corning; 3524) in Matrigel (Corning; 356321) in Advanced MEM/F12 (Invitrogen; 12634-010) supplemented with mEGF (Invitrogen; PMG8041) and Wnt3A, R-spondin, and Noggin conditioned media at 37°C with 5% CO_2. Media was changed every 2 to 3 days and organoids were passaged every 5 to 7 days (Sato & Clevers, 2013). To induce *Cdx2* deletion, 1 µM 4-Hydroxytamoxifen (4-OHT) (Millipore Sigma; H6278-10MG) was incorporated into the media. Four days later, control or *Cdx* DKO organoids were treated with 50 ng/mL TNF-α (Abclonal; RP00993) for 24 hours followed by 10 µM Nigericin (Millipore Sigma; SML1779) for 1 hour. Organoids were then

collected by aspirating the media and adding 1 mL of cold D-PBS directly onto the Matrigel. The mixture was gently resuspended several times until the Matrigel dissolved, and the organoids were transferred to Eppendorf tubes and incubated on ice for 30 minutes. The organoids were centrifuged at 800 x g for 5 minutes, washed with cold D-PBS, and centrifuged again. The cell pellet was resuspended in 500 µL of PBS or 0.1% Triton X-100 (Biotech; 9002-93-1) in PBS to lyse the organoids as a positive control. The organoids were incubated at 37°C for 15 minutes, centrifuged and the supernatant collected for LDH analysis.

LDH assay

The supernatant from control or *Cdx*$^{-/-}$ organoids was transferred into a 96-well plate and the CyQUANT LDH Cytotoxicity Assay Kit (ThermoFisher; C20300) used to assess LDH activity following the manufacturer's protocol. Absorbance was measured using a BioTek Synergy H1 Hybrid Reader at 490 nm and 680 nm and LDH activity determined by subtracting the 680 nm absorbance from the 490 nm value.

Caspase 3/7 assay

Intestinal cells from control or *Cdx*$^{-/-}$ mice were isolated using the TrypLE isolation protocol and resuspended in 300 µL FACS buffer containing 4 mM EDTA. The cells were then incubated for 30 minutes at 4°C with PE-Cy7 CD326 (EpCAM) to identify epithelial cells and Caspase-3/7 Detection Reagent (ThermoFisher, C10432) to evaluate apoptosis. After incubation, the cells were centrifuged as above and washed with FACS buffer containing 4 mM EDTA to remove any unbound antibodies. Cells were resuspended with 500 µL FACS buffer + 4 mM EDTA and flow cytometric analysis was performed on a BD LSR Fortessa. Filters were set to PE-Cy7 (excitation at 488 nm, emission at 780 nm) and Caspase-3/7 reagent (excitation at 488 nm, emission at 530 nm) and compensation was calculated using a negative control and single-stained control samples for each fluorophore. Gating strategies were

applied as described above and epithelial cells that are undergoing apoptosis were analyzed (EpCAM + Caspase-3/7 +).

NLRP3 inhibition

Mice were administered 15 mg/mL of the NLRP3 inhibitor CY-09 (Sigma; SML2465-5MG) or saline intraperitoneally once daily for 5 days and treated on day 2 with 2 mg tamoxifen or corn oil. Animals were euthanized on day 5, and intestinal cells were isolated using the TrypLE protocol. Epithelial (EPCAM+ CD45-) cells were isolated by FACS as describe above and resuspended with RIPA buffer containing protease inhibitor cocktail and 10 mM Sodium Fluoride. 20 µg of protein lysates were then used for Western Blot for Gasdermin D antibody (Table 2), and the results were plotted using GraphPad Prism 10.

Antibody	Species	Application / Dilution	Catalog number	Source
Gasdermin D	Rabbit	WB / 1:1000	PA5-116815	ThermoFisher
β actin	Rabbit	WB / 1:1000	PA1-183	ThermoFisher
Na+/K+ ATPase α-1 Antibody, clone C464.6	Mouse	WB / 1:1000	05-369-25UG	Millipore Sigma
Caspase-1	Rabbit	WB / 1:1000	24232T	Cell Signaling
Goat polyclonal antibody IgG (HRP)	Rabbit	WB / 1:5000	AB7090	Abcam
Goat polyclonal antibody IgG + IgM (HRP)	Mouse	WB / 1:5000	AB47827	Abcam
PE-Cy7 CD326 (Ep-CAM)	Mouse	FACS / 0.25 µg per 10^6 cells in 100 µL volume	118215	BioLegend
PE-CD45	Mouse	FACS / 0.25 µg per 10^6 cells in 100 µL volume	147711	BioLegend
eBioscience 7-AAD Viability Staining	-	FACS / 15 µL	00-6993-50	ThermoFisher

Table 2: Antibodies and Reagents Used for Western Blot, Flow Cytometry, and Cell Sorting Analyses.

Chapter 3

Results

Gasdermin D cleavage is upregulated in *Cdx* mutant intestinal epithelial cells.

GSDMD is a pivotal effector protein in pyroptosis, a form of inflammatory programmed cell death. This process plays a crucial role in innate immunity by eliminating infected or damaged cells and triggering an inflammatory response. Upon NLRP3 inflammasome activation, GSDMD undergoes proteolytic cleavage, releasing its N-terminal fragment. This fragment is responsible for forming transmembrane pores in the lipid bilayer, leading to membrane permeabilization coupled with the release of the pro-inflammatory cytokines IL-1β and IL-18, which are central mediators of inflammation and immune signaling. The subsequent lytic cell death further amplifies the inflammatory response by releasing intracellular contents into the extracellular environment.

Our prior work (Jahan et al., 2022) demonstrated that inflammation is significantly increased in *Cdx* mutant mice. RNA-Seq analyses and subsequent RT-qPCR validation revealed that the expression of cytokine and chemokine genes, including *TNF-α, IL-1β, CXCL1, CCL2* and the NLRP3 inflammasome is upregulated in the *Cdx* double-knockout intestine. These findings suggest a heightened inflammatory state in the *Cdx* mutant intestinal environment, potentially driven by increased inflammasome activity. However, little is known about GSDMD processing and the role of pyroptosis in the intestinal epithelium. This highlights the need for further investigation into the mechanisms linking inflammasome activation, GSDMD processing, and pyroptosis in the context of intestinal inflammation.

I began investigating the effect of *Cdx* loss on GSDMD by comparing its expression in control and *Cdx* mutant mice. To establish these models, mice were treated with either oil or 2 mg of tamoxifen, respectively, for 4 days before isolating intestinal cells for analysis. Methods for isolating intestinal cells

from mice presented significant challenges. The complexity of the intestinal structure, the delicate nature of epithelial cells, and, in particular, the need to maintain cell viability made the process particularly demanding. Initial attempts, which involved high temperatures and vigorous shaking, frequently led to low yields and/or poor cell viability. This was either due to harsh dissociation methods that caused cell damage or inadequate enzymatic digestion, which left tissue behind.

One of the key challenges was identifying a protocol that effectively dissociated the intestinal epithelium while preserving the integrity and viability of single cells. Eventually, I identified the TrypLE-based protocol (Ge et al., 2023) as a suitable method. This protocol involved isolating intestinal cells using 30 mM EDTA in PBS, followed by dissociation into single cells using TrypLE Express Enzyme. Compared to other methods, the TrypLE protocol consistently yielded a higher number of viable cells (typically greater than 90%), making it suitable for downstream applications such as cell sorting, flow cytometry and Western Blotting.

After isolating intestinal cells from both control and *Cdx* mutant mice, Western blot analysis was used to measure the levels of both full length GSDMD (60 kDa) and cleaved NT-GSDMD (25 kDa) using a GSDMD polyclonal antibody. The results **(**Figure 10) show clear differences in GSDMD cleavage between control and *Cdx* mutant intestinal cells. The NT-GSDMD, which represents the active form, was more abundant in *Cdx* mutant cells compared to control cells (Figure 10A), with quantitative analysis indicating a 3.7-fold higher level in the mutants (Figure 10C). This suggests that more GSDMD is being processed into its active form in the mutant cells, consistent with elevated NLRP3 levels following *Cdx* deletion (Jahan et al., 2022). Also in agreement with this, control cells exhibited approximately 6 fold higher levels of full length GSDMD than *Cdx* mutant cells (Figure 10A and 10B). This indicates that in WT cells, a greater proportion of GSDMD remains in its unprocessed state.

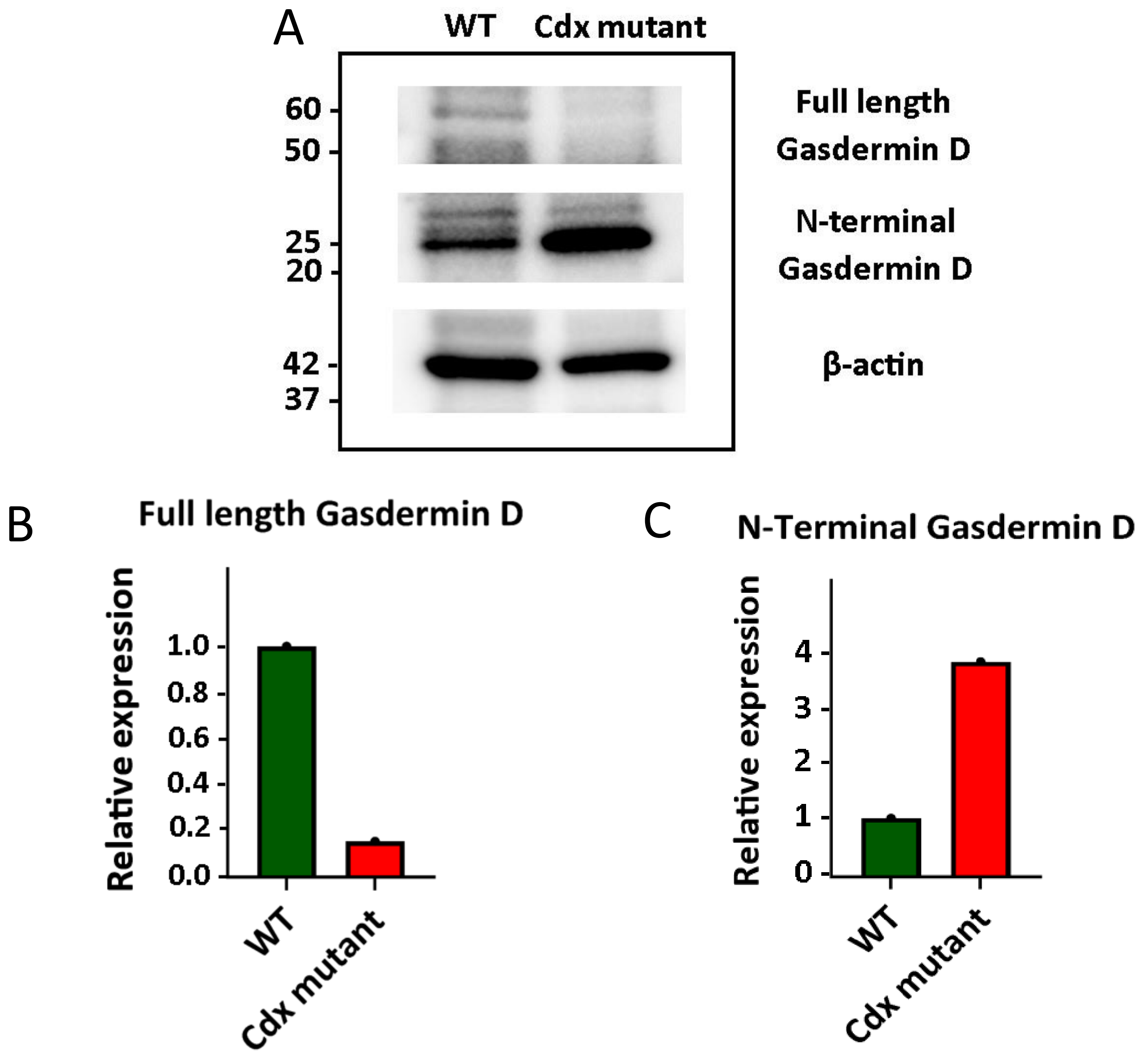


Figure 10: **Gasdermin D expression is elevated in the intestinal cells of *Cdx* mutant mice.** (A) Immunoblot analysis showing full length and cleaved NT-GSDMD in WT and *Cdx* mutant intestinal cell lysates. β-actin was used as a loading control. Quantification of relative amounts of (B) full length GSDMD and (C) NT-GSDMD expression in WT and *Cdx* mutant intestinal cells.

Gasdermin D processing is increased in the epithelial cell compartment of *Cdx* mutant mice.

Based on results indicating an increase in the cleaved form of GSDMD in *Cdx* mutant cells, I proceeded to focus on analyzing GSDMD expression in epithelial cells. The intestine is a complex tissue made up of various cell types, including epithelial cells (enterocytes, goblet cells, Paneth cells, M cells and intestinal stem cells) and infiltrating cells, primarily immune cells. Therefore, means to isolate intestinal epithelial cells was necessary to prevent contamination by non-epithelial cells, which could produce confounding results. To accomplish this, control and *Cdx* mutant intestinal cells were first isolated using the TrypLE protocol as described in Materials and Methods. After isolation, the cells were stained with fluorophore-coupled antibodies against EPCAM (epithelial cells) and CD45 (immune cells) with viability assessed by inclusion of 7-AAD. Epithelial cells, identified as EPCAM-positive and CD45-negative, were then isolated using fluorescence-activated cell sorting (FACS) on a MoFlo system.

After sorting WT and *Cdx* mutant epithelial cells, a Western Blot was performed on lysates from independent triplicate samples and probed with GSDMD antibody. As seen in Figure 11A, there was an increase in NT-GSDMD in the *Cdx* mutant epithelial cells compared to the control, with the relative level of NT-GSDMD in the mutant cells approximately 14 times higher than in the control group (Figure 11B). Note that full-length GSDMD could not assessed due to non-specific binding to the antibodies used to sort the epithelial cells in the range of 50 to 75 kDa, which overlaps with the expected molecular weight of full length GSDMD (60 kDa). This interference made it impossible to assess relative amounts of full-length GSDMD and to compare it between WT and *Cdx* mutant epithelial cells. These findings, however, suggest that GSDMD may play a significant role in epithelial cells, in addition to its well-known function in pyroptosis in immune cells during inflammation (J. Shi et al., 2015). These results also highlight the importance of Cdx in regulating GSDMD function, consistent with its role in epithelial integrity (Hinoi et al., 2002; Sakaguchi et al., 2002). In addition, the upregulation of GSDMD in *Cdx*

mutant epithelial cells is consistent with the induction of NLRP3 in *Cdx* mutant mice (Jahan et al., 2022).

Gasdermin D processing is dependent on NLRP3 activity in epithelial cells.

Previous work from our lab demonstrated that the NLRP3 inflammasome is upregulated in *Cdx* mutant intestinal cells, along with the inflammasome products, IL-1β and IL-18, indicating that Cdx regulates NLRP3 inflammasome activity. Additionally, our lab identified *TRIM31*, a NLRP3 ubiquitin ligase, as a direct target gene of Cdx, and proposed a Cdx2-TRIM31-NLRP3 pathway that regulates the production and release of pro-inflammatory cytokines. Importantly, numerous studies have shown that the NLRP3 inflammasome can also regulate GSDMD activity. This suggests a potential link between NLRP3-mediated cytokine release and GSDMD activation in the inflammatory response, suggesting an interplay between these pathways in modulating intestinal inflammation. Therefore, I investigated whether NLRP3 plays a similar role for GSDMD processing in epithelial cells by blocking the NLRP3 inflammasome with a specific direct inhibitor.

To determine the impact of NLRP3 activity in *Cdx*-dependent GSDMD processing, mice were treated with the NLRP3 inhibitor CY-09 or saline as control and *Cdx2* deletion initiated with tamoxifen. After 5 days, cells were isolated using the TrypLE protocol, and a Western blot was conducted. A significant reduction was observed in the N-terminal form of Gasdermin D (NT-GSDMD) in *Cdx* mutant cells receiving CY-09 compared to the untreated conditional mutants (Figure 11A) being reduced by approximately 14-fold in the cells ($p < 0.05$) (Figure 11B). These results show that GSDMD cleavage depends on the NLRP3 inflammasome activity.

Cell sorting analysis was performed on $EpCAM^{+}$ $CD45^{-}$ cells to assess 7-AAD positivity, a marker of cell death. 7-AAD is a membrane-impermeable dye that selectively binds to DNA in cells with

compromised membrane integrity, fluorescing to indicate cell death (Zimmermann & Meyer, 2011). The comparison between untreated *Cdx* mutant cells and *Cdx* mutant cells treated with CY-09 revealed a significant decrease in cell death, approximately 3-fold (Figure 11C). These results demonstrate that inhibition of the NLRP3 inflammasome by CY-09 also provided protection against cell death consistent with the suppression of GSDMD activation, which is critical for pyroptotic cell lysis.

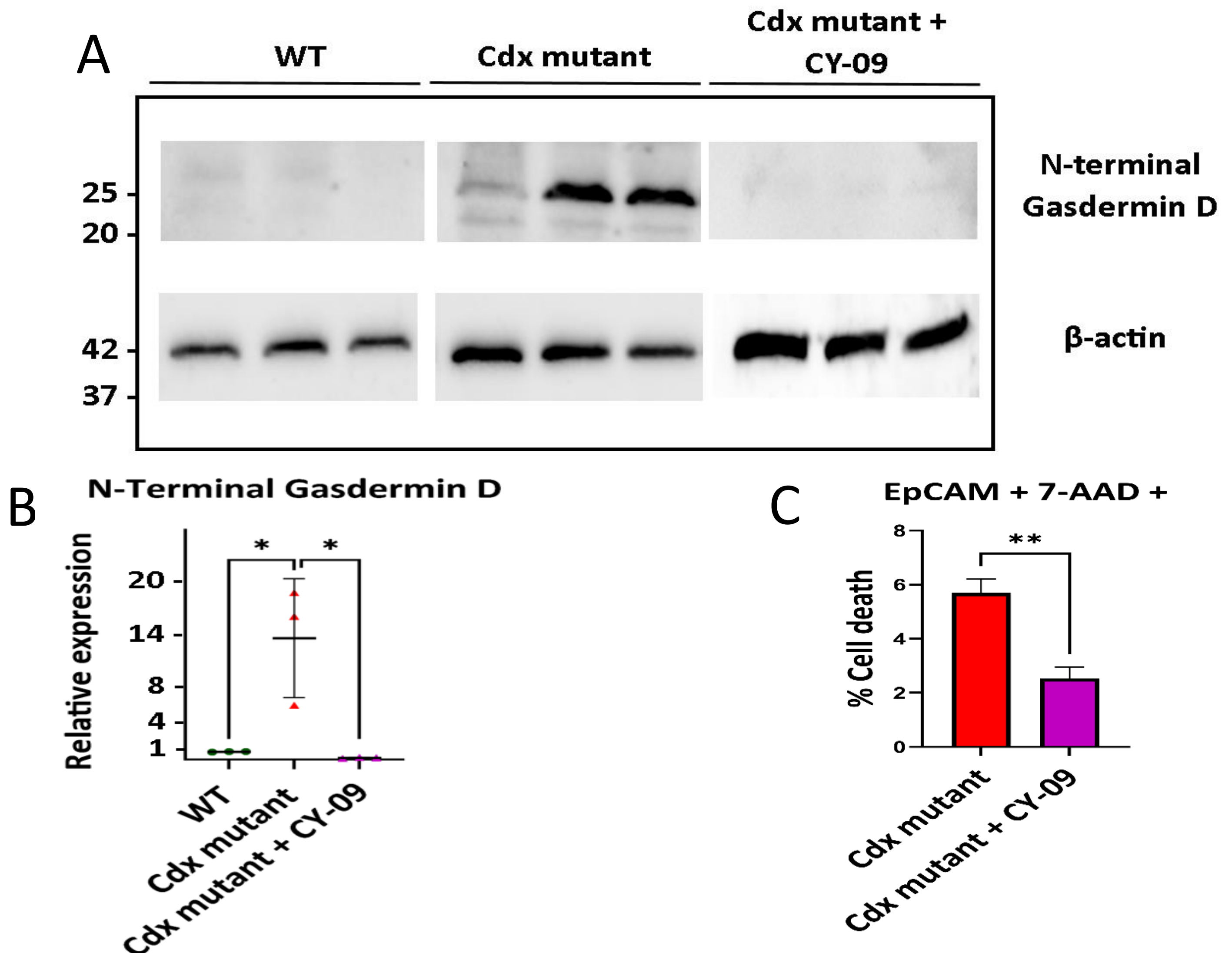


Figure 11: **Elevated N-terminal Gasdermin D in *Cdx* Mutant Epithelial Cells is Dependent on NLRP3 Activity.** (A) Immunoblot analysis showing NT-GSDMD in WT, *Cdx* mutant and *Cdx* mutant mice treated with CY-09. β-actin was used as a loading control. (B) Quantification of relative NT-GSDMD expression in WT, *Cdx* mutant and *Cdx* mutant intestinal cells treated with CY-09. (C) Bar graph showing the percentage of dead cells in *Cdx* mutant and *Cdx* mutant epithelial cells treated with CY-09. Error bars represent the standard deviation from the mean of three independent biological mice. Statistical significance was calculated using two-tailed t test, $*p < 0.05$ and $**p < 0.01$.

Gasdermin D cleavage is associated with cell death in *Cdx* mutant intestinal cells.

Cell death is a fundamental biological process that can occur through several distinct mechanisms, each with unique molecular and morphological features. Apoptosis, or programmed cell death, is a highly regulated and non-inflammatory process essential for tissue homeostasis and development. It is characterized by the activation of caspases, a family of proteases that orchestrate the orderly dismantling of cellular components. Morphologically, apoptosis is associated with chromatin condensation, DNA fragmentation, cell shrinkage, and the formation of apoptotic bodies, which are then phagocytosed by neighboring cells (Elmore, 2007).

Pyroptosis, in contrast, is an inflammatory form of programmed cell death typically initiated by the activation of inflammasomes, such as the NLRP3 inflammasome, in response to microbial infections or cellular stress. This process leads to the cleavage of Gasdermin D by caspase-1, which forms membrane pores that result in osmotic lysis and the release of pro-inflammatory cytokines such as IL-1β and IL-18. Morphologically, pyroptotic cells exhibit swelling, membrane rupture, and leakage of cellular contents which contribute to local and systemic inflammatory responses. Other forms of cell death exists such as necrosis which is often associated with uncontrolled cell damage resulting in membrane disruption and inflammation (D'Arcy, 2019).

To address mechanisms of cell death evoked by *Cdx* loss, sorting data were analyzed, focusing on EpCAM-positive, CD45-negative, 7-AAD-positive cells. This analysis revealed that *Cdx* mutant cells displayed approximately double the level of 7-AAD-positive cells compared to control cells, as shown in Figure 12A. This suggests a marked increase in epithelial cell death in the mutants, consistent with increased inflammatory signaling and cellular stress associated with the *Cdx* mutant intestinal phenotype (Chewchuk et al., 2021; Jahan et al., 2022).

To further evaluate cell death, an LDH (lactate dehydrogenase) assay was performed. LDH is a cytosolic enzyme released when the cell membrane is damaged, serving as a marker of cell injury or death. In this assay, the amount of LDH released into the culture medium is measured based on its ability to convert a substrate into a detectable product (Chan et al., 2013). A higher level of LDH in the supernatant reflects greater cell damage or death.

Conducting the LDH assay on sorted epithelial cells was not feasible due to the potential for a high background caused by the considerable baseline of cell death during sorting (Figure 12A). As an alternative, this assay was conducted using *Cdx* conditional mutant colon organoids. Organoids are ideal for this experiment as they are a pure population representing the intestinal epithelium and eliminating the need for sorting. Colon organoids were generated from intestinal tissues of *Cdx1*$^{-/-}$ *Cdx2*$^{f/f}$ *Villin Cre ERT* mouse and *Cdx2* deletion was effectively induced using 4-Hydroxytamoxifen (4-OHT).

Cells were treated with either vehicle or 1 µM 4-OHT. After 4 days, when *Cdx2* deletion was largely achieved (unpublished observation, our lab), the cells were collected using cold PBS to dissolve the Matrigel, incubated in PBS at 37°C for 20 minutes, centrifuged, and the supernatant collected for the LDH assay. Cells treated with Triton X-100 in PBS for 20 minutes at 37°C, which causes immediate cell lysis, were used as a positive control.

The results showed that both vehicle and tamoxifen-treated groups had similar levels of cell death, which contradicted expectations, as well as findings from epithelial cells (Figure 12A). In this regard, recent work from our group suggests that these organoids do not express detectable levels of NLRP3 by Western blot, either at baseline or following deletion of *Cdx2*. This absence of NLRP3 led us to conclude that they are not a suitable model for this analysis.

To show further assess mechanisms of cell death, I assessed caspase 3/7 activity in control and *Cdx* mutant intestinal cells using flow cytometry. Apoptosis, or programmed cell death, is a critical process

in maintaining tissue homeostasis, and dysregulation of this process can contribute to diseases such as cancer. Caspases 3 and 7 are key executioner caspases in this pathway, responsible for cleaving a variety of cellular proteins, including Poly (ADP-ribose) polymerase (PARP), vimentin, and Inhibitor of Caspase-activated DNase (ICAD), which leads to cell dismantling (Walsh et al., 2008). These caspases are activated via intrinsic or extrinsic pathways. The assay quantifies the fluorescence generated when active caspase 3/7 cleaves specific reporter substrates, producing a fluorogenic signal (ThermoFisher Scientific). The intensity of this fluorescence directly correlates with caspase activity, allowing for the quantification of apoptosis in a population of cells.

Intestinal cells from WT and *Cdx* mutant mice were isolated, then incubated with caspase 3/7 detection reagent and EpCAM antibody to identify epithelial cells by flow cytometry. The results revealed only a 5% increase in reporter-positive *Cdx* mutant cells relative to controls (Data not shown). This suggests that induction of apoptosis is not the dominant form of cell death in the mutant cells, rather the relatively low difference in apoptotic cells, along with the fact that 7-AAD staining identifies cells with compromised membrane integrity, raises the possibility that other forms of cell death are involved. Given that pyroptosis is characterized by inflammasome activation and the subsequent formation of pores by N-terminal GSDMD, I hypothesized that the observed cell death could be partially attributed to pyroptosis.

However, the 5% increase in apoptosis is also noteworthy, and further investigation into the specific contributions of both apoptosis and pyroptosis is warranted to better understand the mechanisms underlying cell death in the *Cdx* mutant cells.

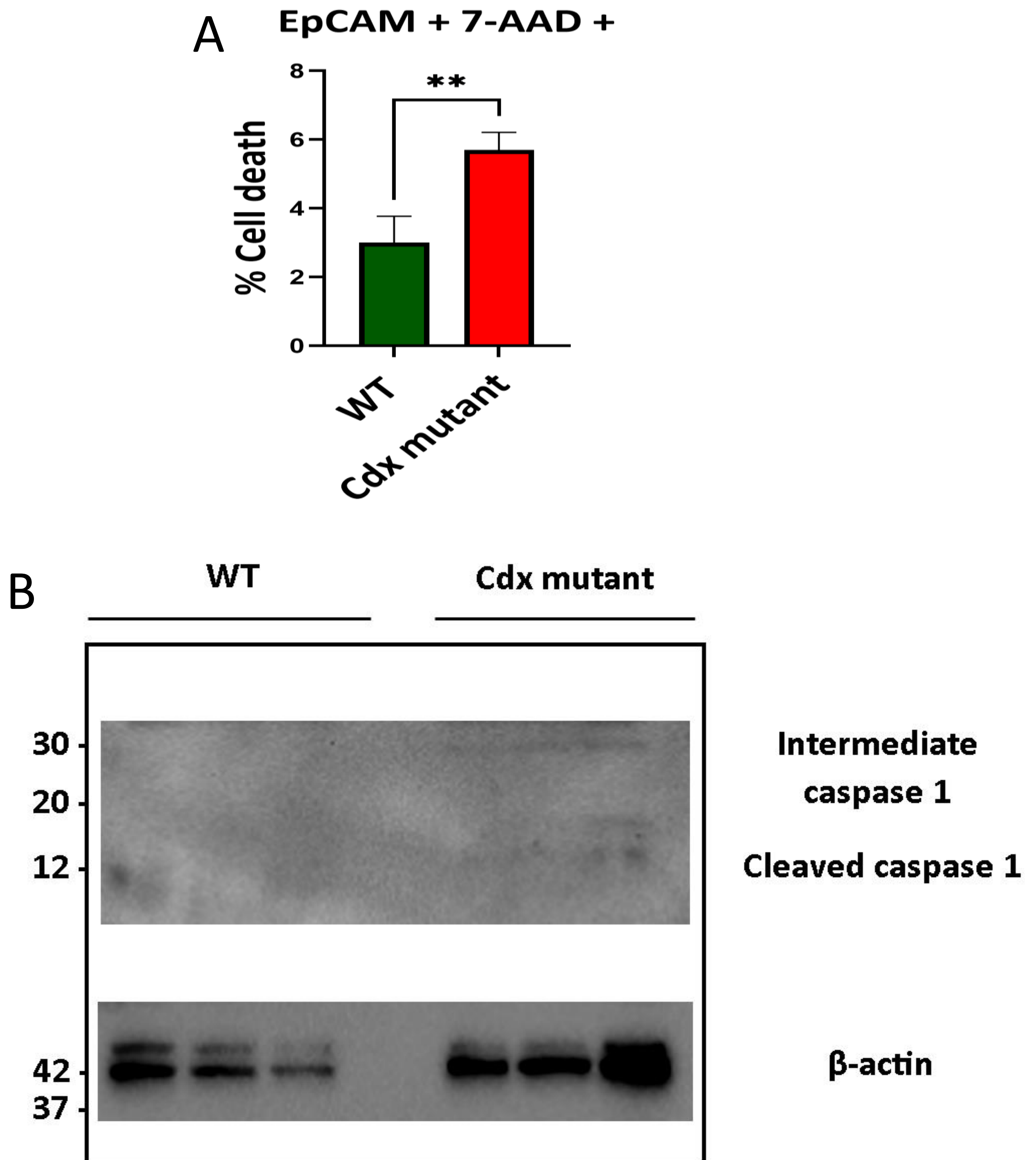
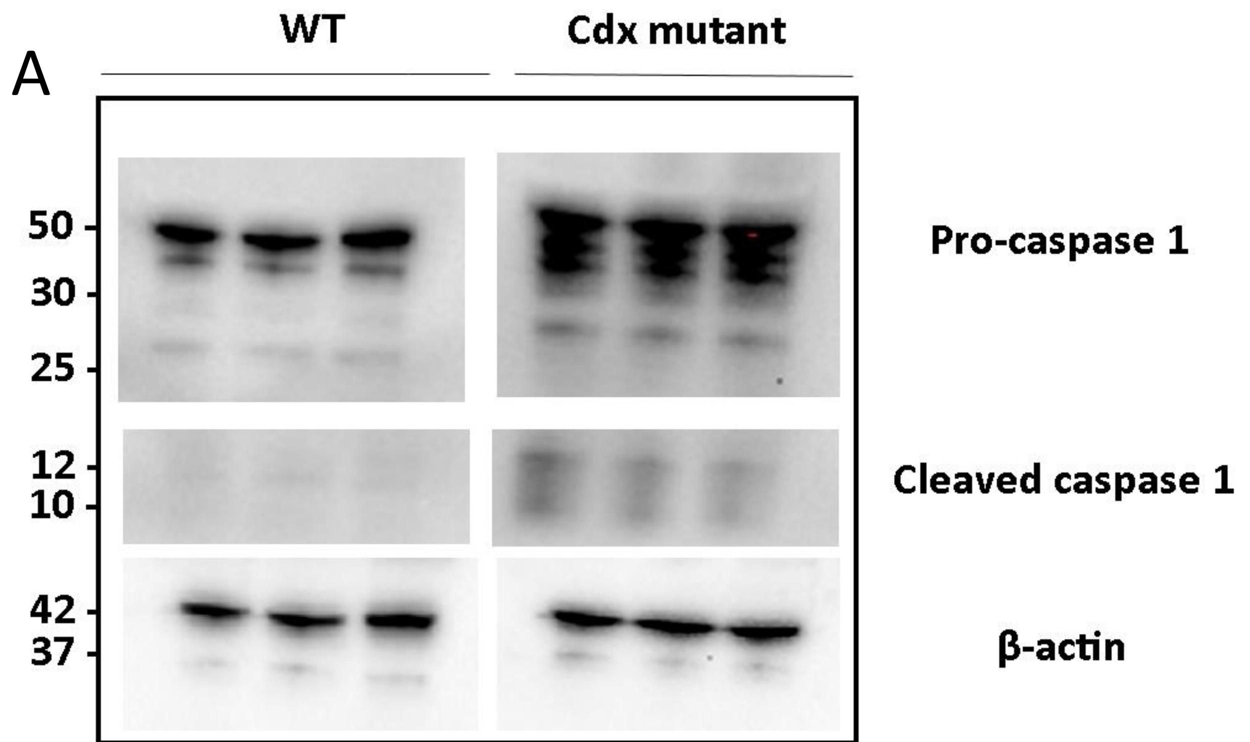


Figure 12: ***Cdx*** **deletion increases cell death.** Bar graphs showing the percentage of (A) dead cells in WT and *Cdx* mutant epithelial cells. Error bars represent the standard deviation from the mean of three independent biological mice. (B) Immunoblot analysis showing active and cleaved caspase 1 in WT and *Cdx* mutant sorted epithelial cell lysates. β-actin was used as a loading control. Statistical significance was calculated using two-tailed t test, $^{**}p < 0.01$.

Caspase-1 is critical for pyroptosis, and, as previously described, leads to the cleavage of Gasdermin D. Caspase-1 activation can therefore be used as a relative measure of pyroptosis. As shown in Figure 12B, activation of pro-caspase 1 is evident in *Cdx* mutant epithelial cells, with the appearance of both the intermediate (30 kDa) and cleaved (12 kDa) forms of the protein. In contrast, control samples had only minimal evidence of processing.

Although caspase 1 cleavage in *Cdx* mutant cells was observed, the intensity of the signal was relatively weak. In this regard, studies have suggested that caspase-1 processing is more reliably detected from extracellular supernatants, as active caspase-1 is known to be released via a secretion pathway that has not yet been fully characterized (Broz et al., 2010). This release of active caspase 1 into the surrounding medium could account for low level of detection of the cleaved forms of the enzyme within the cellular lysates.

To address this issue, control and *Cdx* mutant intestinal explants were collected and incubated in DMEM for 2 hours at 37 °C in 5% CO_2. After incubation, the media was collected and centrifuged at 500 × g for 5 mins and used for Western Blot analysis. As shown in Figure 13A, the supernatant from *Cdx* mutant explants contained cleaved caspase-1 (12 kDa), representing the active form of the enzyme. In contrast, minimal cleaved caspase-1 was observed in the control supernatant. Notably, cleaved caspase-1 levels were approximately 5-fold higher in *Cdx* mutant cells compared to control cells (Figure 13B). This suggests that the caspase-1 activated at higher levels in the *Cdx* mutant cells and that it is predominantly released into the extracellular environment.



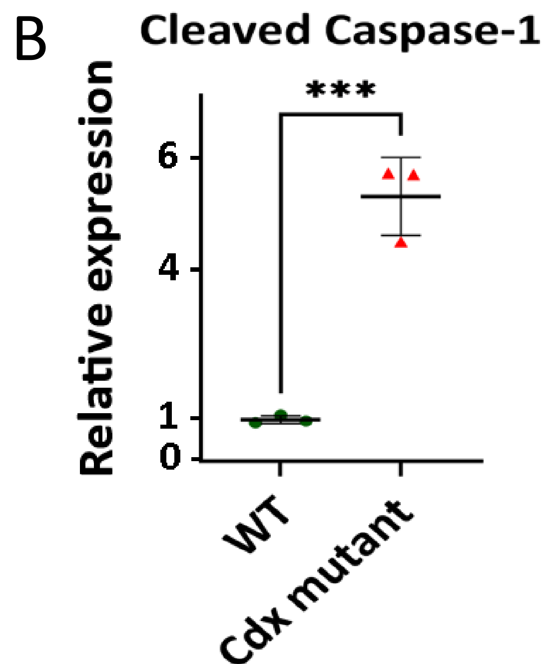


Figure 13: **Caspase-1 is activated in *Cdx* mutant intestinal explants.** (A) Immunoblot analysis showing unprocessed and cleaved caspase 1 in WT and *Cdx* mutant intestinal explants supernatant. β-actin was used as a loading control. (B) Quantification of immunoblot results of cleaved caspase 1. Error bars represent the standard deviation from the mean of three independent biological mice. Statistical significance was calculated using two-tailed t test, ***$p < 0.001$.

The N-terminal fragment of Gasdermin D translocates from the cytoplasm to the membrane in *Cdx* mutant epithelial cells.

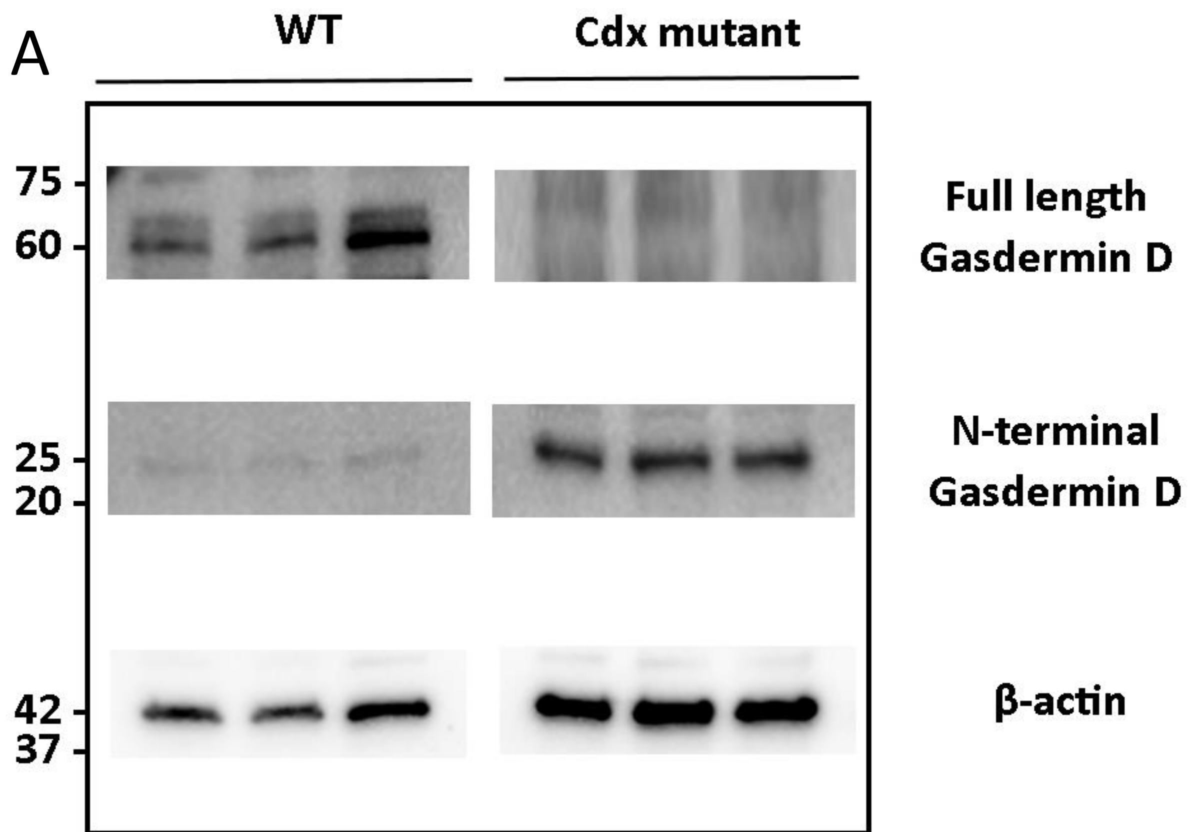
Once cleaved, the N-terminal fragment of GSDMD undergoes a conformational change, moving from the cytoplasm to the plasma membrane, where it integrates into the membrane. The N-terminal fragment subsequently oligomerizes, forming large, stable pores that compromise cellular integrity and trigger pyroptotic cell death. To investigate whether this translocation process occurs in *Cdx* mutant epithelial cells, cytoplasmic and membrane protein fractions were isolated by subcellular fractionation. Following ultracentrifugation at 100,000 x g for 1 hour, the supernatant (cytoplasmic fraction) was collected, while the pellet (membrane fraction) was resuspended in Laemmli buffer and both fractions assessed for GSDMD by Western blot.

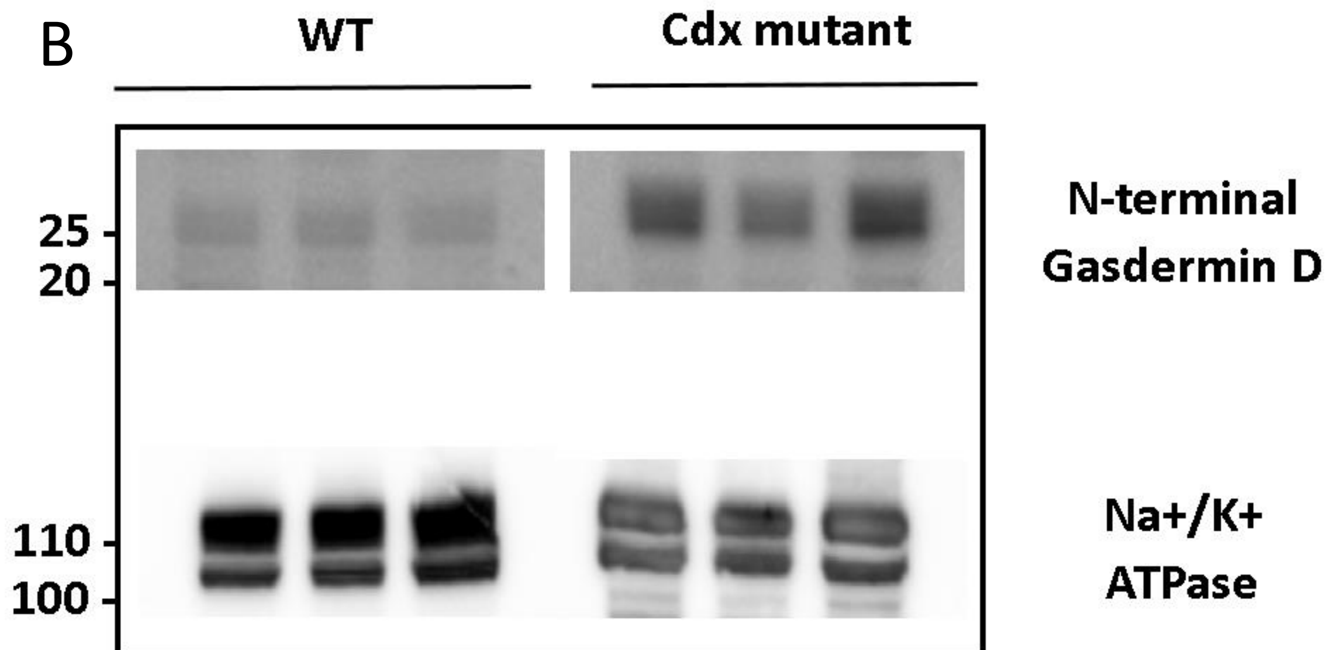
I initially attempted to perform subcellular fractionation on sorted control and *Cdx* mutant cells, but encountered challenges due to the low yields of the membrane fraction, likely due to the low number of sorted cells, which made it difficult to obtain sufficient protein. Given this limitation, I decided to perform the fractionation on unsorted intestinal cells, which provided a larger sample for subsequent analysis.

The results revealed significant differences in GSDMD processing and distribution between control and *Cdx* mutant cells. *Cdx* mutant cells exhibited much higher levels of cleaved cytoplasmic GSDMD compared to control cells, which retained more of the full-length form of the protein (Figure 14A, C, D). Consistent with this, Western blot analysis further demonstrated a 34-fold increase in the GSDMD N-terminal fragment associated with the membrane fraction in *Cdx* mutant cells compared to control (Figure 14B, E). This suggests that the membrane-associated and oligomerized form of GSDMD is significantly more abundant in the mutant cells potentially driving increased pyroptosis.

Although this experiment was conducted on total intestinal cells rather than sorted cells, the data strongly support GSDMD translocation from the cytoplasm to the membrane in epithelial cells. This is evident from the complete absence of full length GSDMD in the cytoplasmic fraction of *Cdx* mutant cells, strongly suggesting that GSDMD is being cleaved and processed into its active form in all cell types, allowing it to translocate to the membrane to form pores and promoting pyroptosis-mediated cell death.

To assess the purity of the cytoplasmic and membrane fractions during the analysis of GSDMD, I performed a reblotting experiment using specific marker proteins for each fraction. The membrane fraction was reblotted with β-actin, a cytoplasmic marker, while the cytoplasmic fraction was reblotted with Na+/K+ ATPase, a well-established membrane marker. This dual reblotting approach was critical for interpreting the analysis of GSDMD distribution. Although there was a modest degree of contamination observed in both fractions (Figure 15A, B), it was predominantly detected in the control lysates and does affect the interpretation of the results.


A
WT
Cdx mutant
75
60
Full length
Gasdermin D
25
20
N-terminal
Gasdermin D
42
37
β-actin


B
WT
Cdx mutant
25
20
N-terminal
Gasdermin D
110
100
Na+/K+
ATPase

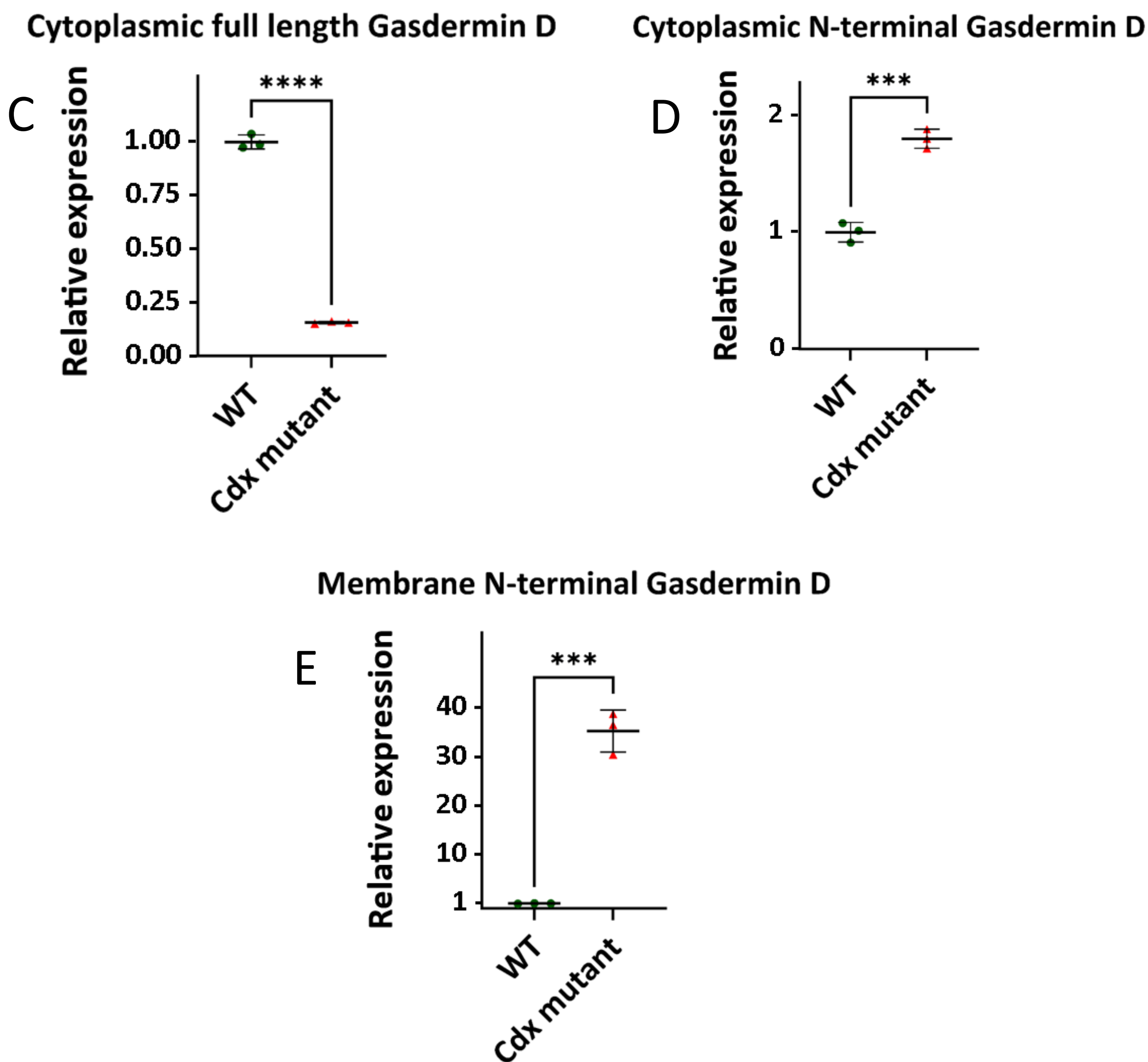


Figure 14: **The N-Terminal Gasdermin D translocates from the cytoplasm to the membrane in *Cdx* mutant epithelial cells.** Immunoblot analysis shows GSDMD in (A) cytoplasmic and (B) membrane fractions for WT and *Cdx* mutant cells, using β-actin as a loading control for cytoplasmic fractions and Na+/K+ ATPase for membrane fractions. Quantification of Gasdermin D from the immunoblot results is shown for (C, D) cytoplasmic and (E) membrane fractions. Error bars represent the standard deviation from the mean of three independent biological mice. Statistical significance was calculated using two-tailed t test, ***$p < 0.001$ and ****$p < 0.0001$.

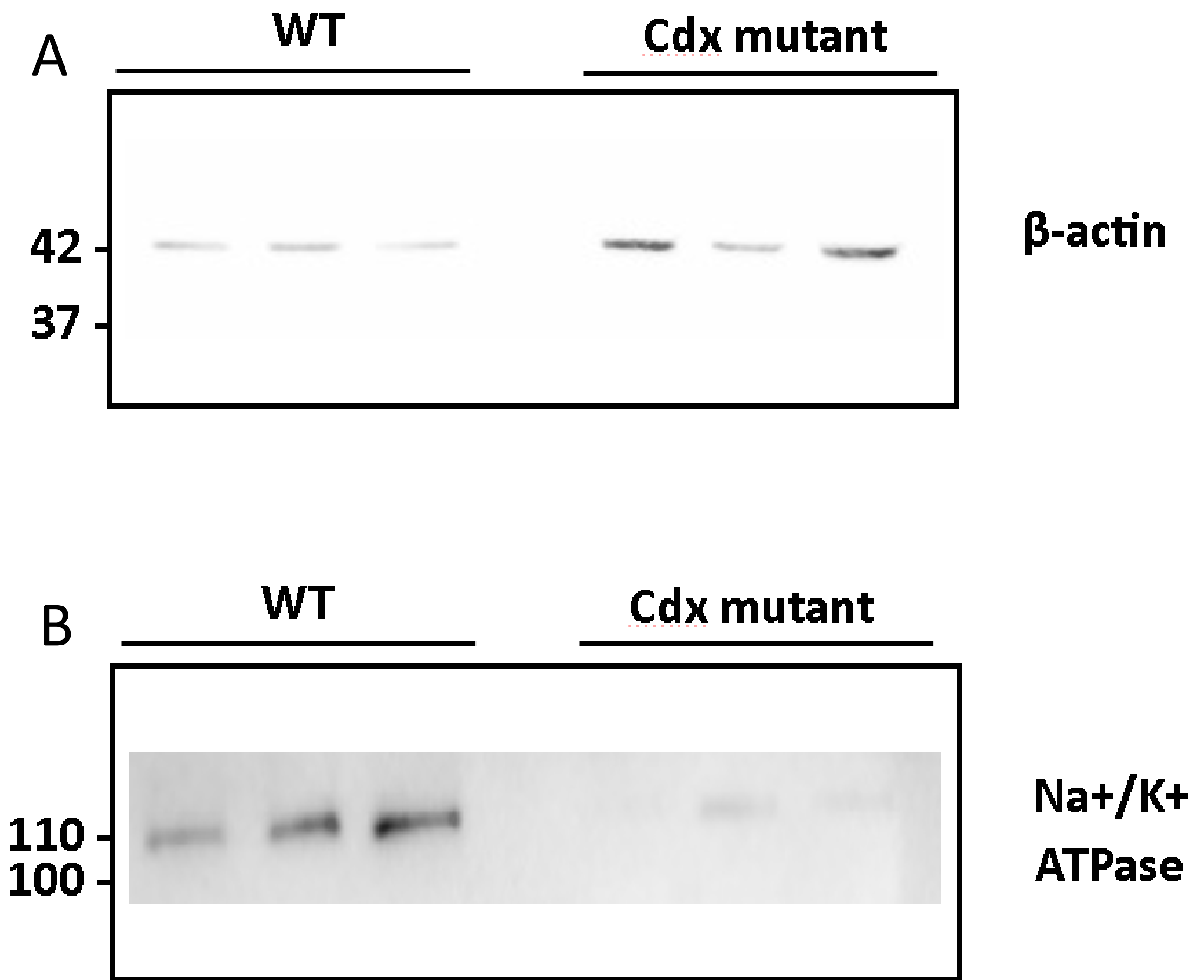


Figure 15: **Purity assessment of cytoplasmic and membrane protein fractions.** Immunoblot analysis includes a reprobing of (A) the membrane fraction with for β-actin and (B) the cytoplasmic fraction for Na+/K+ ATPase to assess the purity of the cytoplasmic and membrane fractions used for GSDMD analysis.

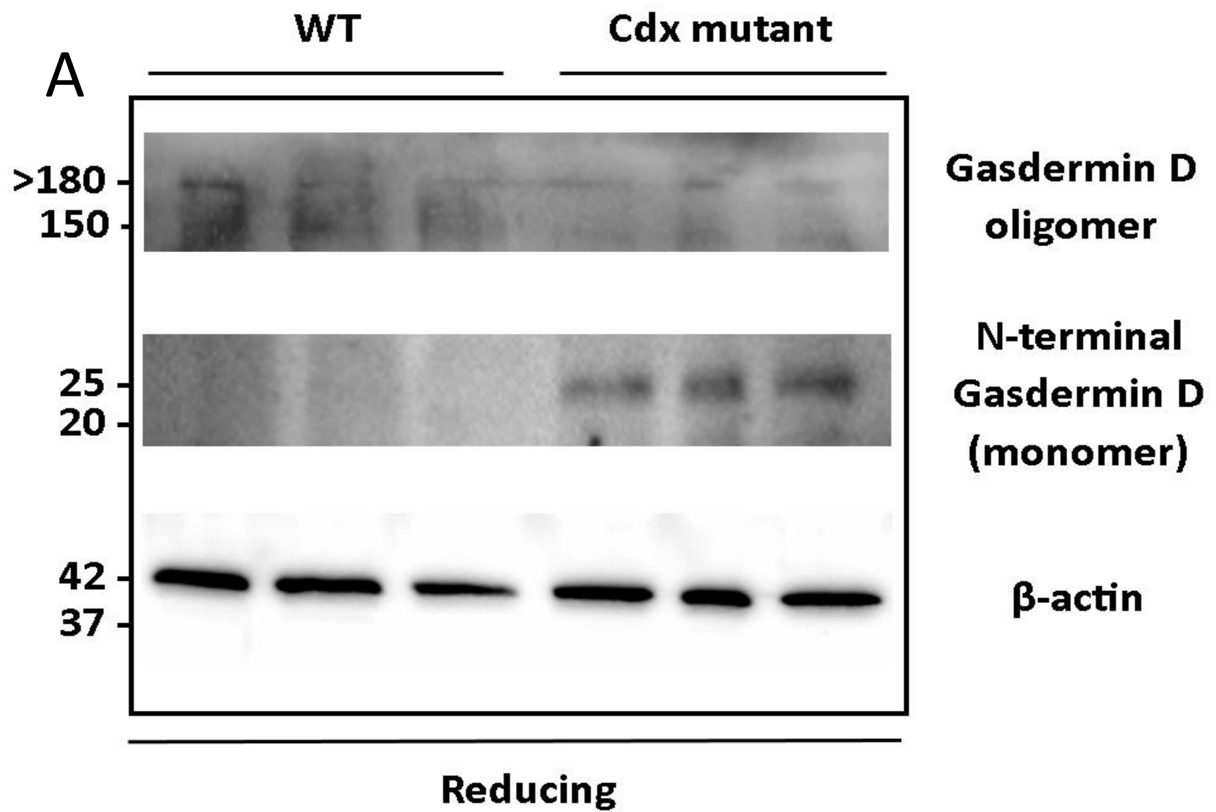
Gasdermin D oligomerizes in *Cdx* mutant epithelial cells.

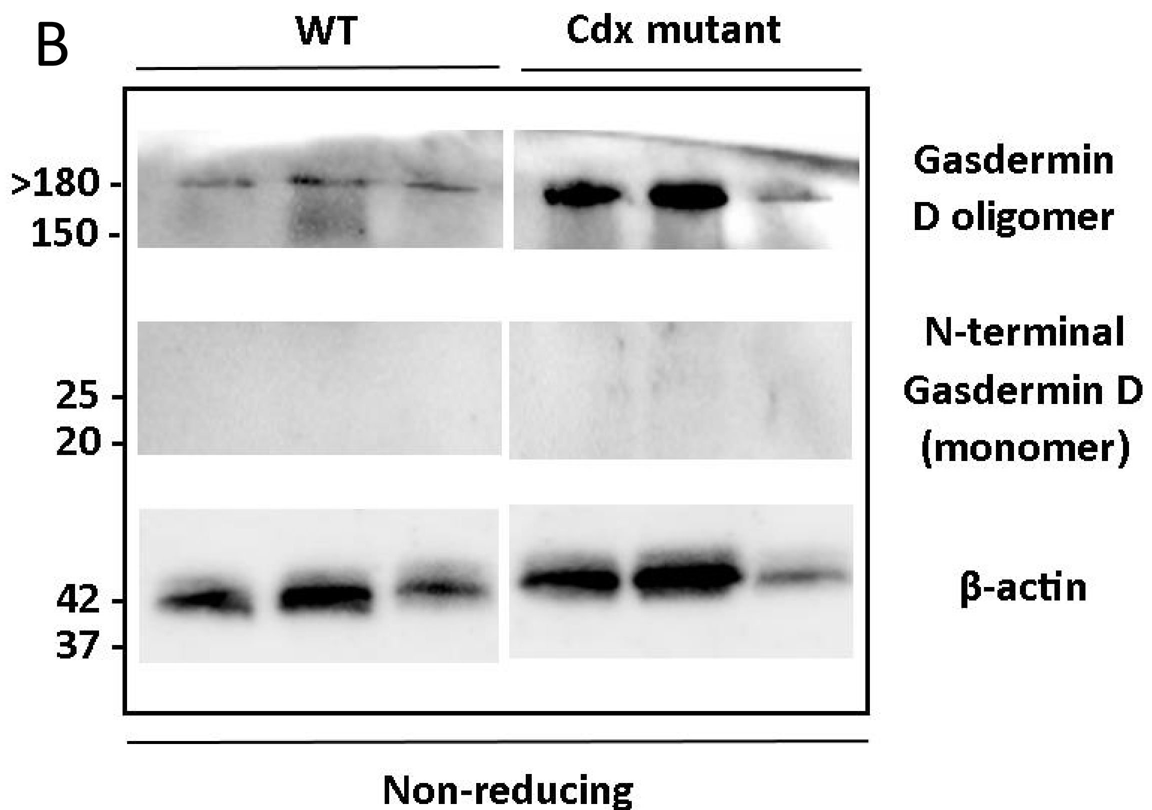
After GSDMD is cleaved and translocates to the membrane, the NT-GSDMD oligomerizes and form pores, triggering pyroptosis. To investigate if this oligomerization occurs in *Cdx* mutant cells, the electrophoretic behaviour of NT-GSDMD under both reducing and non-reducing conditions was examined (Devant & Kagan, 2023). Under non-reducing conditions, NT-GSDMD remains in large oligomers, mediated by formation of disulfide bonds and other native interactions, which are necessary to keep it in a pore-forming state. When a reducing agent such as β-mercaptoethanol is added, the disulfide bonds in the GSDMD oligomers are disrupted, causing the oligomers to shift from large complexes to monomers.

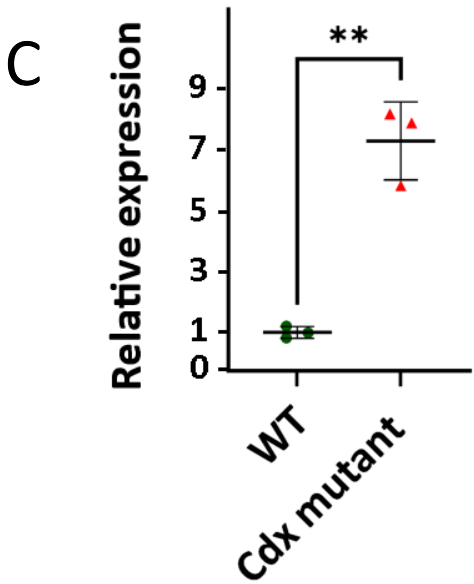
To assess this, the migratory properties of GSDMD from control and *Cdx* mutant sorted epithelial cells were compared under both conditions. Under reducing conditions, using β-mercaptoethanol in Laemmli buffer, GSDMD bands at 25 kDa were observed from *Cdx* mutant epithelial cells (Figure 16A, C), indicating the presence of GSDMD N-terminal monomers, while no such bands were detected in the control cells, consistent with minimal cleavage. In contrast, under non-reducing conditions, GSDMD oligomers were observed migrating at greater than 180 kDa in isolates from *Cdx* mutant epithelial cells, but such complexes were minimal in extracts from control cells (Figure 16B, D).

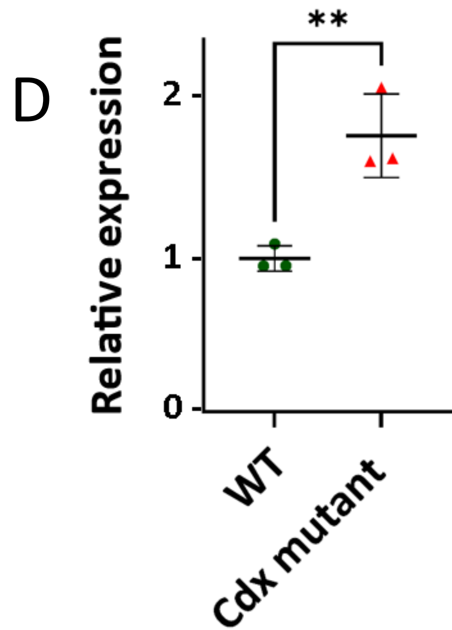
To improve the visualization of GSDMD oligomers in the *Cdx* mutant, 4 M urea was incorporated into the Laemmli buffer. Urea disrupts hydrogen bonds and denatures proteins, ensuring the reduction of large oligomeric structures. This allows for more uniform migration of the proteins during electrophoresis. As a result, a distinct oligomeric ladder was observed in isolates from *Cdx* mutant epithelial cells, indicating dissociation of GSDMD complexes (Figure 16E). This ladder includes the monomer (~25 kDa), representing the N-terminal fragment, as well as higher molecular weight bands indicative of mulitmers of the monomeric form (~50, 75, 100 kDa, etc.). These findings suggest that

GSDMD is oligomerized to form pore structures in the membrane in *Cdx* mutant cells while it remains unprocessed in control cells.









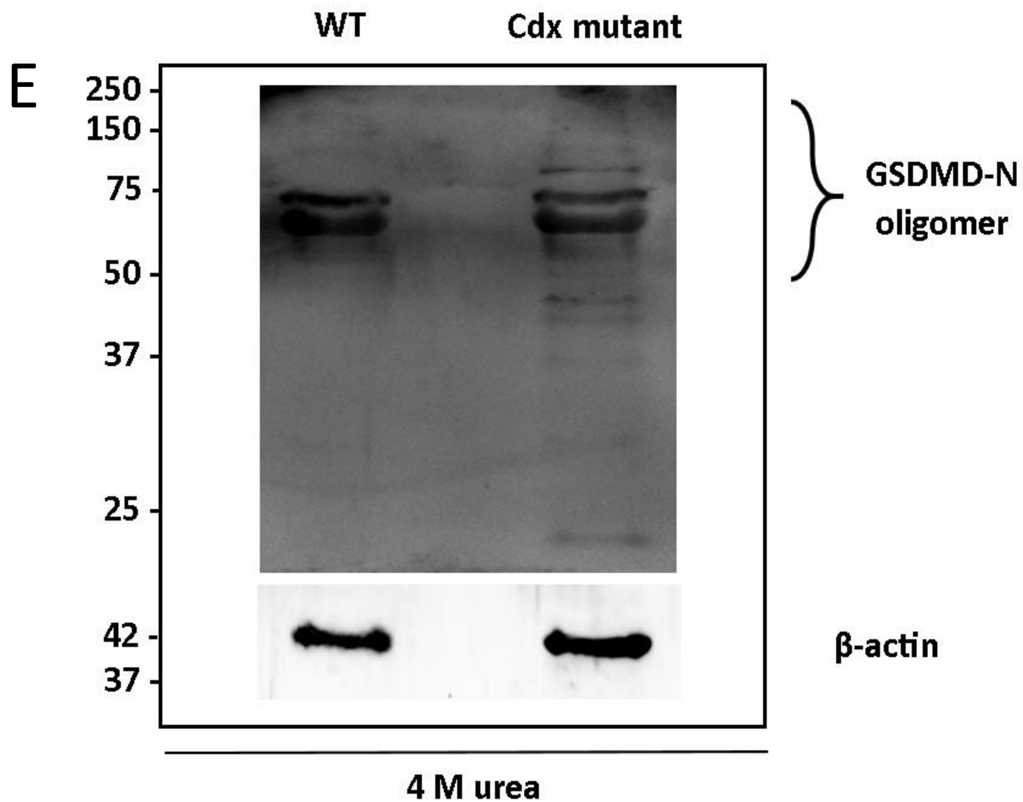


Figure* 16*: **Gasdermin D undergoes oligomerization in *Cdx* mutant epithelial cells.** Immunoblot analysis showing GSDMD oligomers and monomers in lysates from WT and *Cdx* mutant sorted epithelial cells under (A) reducing and (B) non-reducing conditions. β-actin was used as a loading control. Quantification of immunoblot results in (C) reducing and (D) non-reducing conditions. (E) Immunoblot analysis showing GSDMD in WT and *Cdx* mutant sorted epithelial cells with samples prepared using Laemmli buffer supplemented with 4 M urea. Error bars represent the standard deviation from the mean of three independent biological mice. Statistical significance was calculated using two-tailed t test, $^{**}p < 0.01$.

Chapter 4

Discussion

IBD is a chronic condition affecting millions of people globally, leading to significant physical, emotional, and economic burdens. Advances in research have reshaped our understanding of the complex etiology of IBD, which involves genetic, environmental, and immune factors, and the gut microbiota. A hallmark of IBD is the disruption of the intestinal epithelial barrier, a critical structure that maintains gut homeostasis and prevents excessive immune activation (Qiu et al, 2022).

Among the key regulators of intestinal homeostasis are the *Caudal*-related homeobox (*Cdx*) genes, particularly *Cdx2*. Using a conditional knockout model to circumvent early lethality associated with complete knockout, *Cdx2* has been shown to be essential for maintaining intestinal patterning and epithelial cell differentiation, as well as regulating barrier integrity. In addition to these roles, Cdx2 also modulates intestinal inflammation potentially by regulating the expression of several IBD-susceptibility genes (Banerjee et al., 2009; Barrett et al., 2008; Coskun, 2014; Coskun et al., 2011, 2017; Moehle et al., 2006).

Recent studies from our lab have identified a potential regulatory axis involving Cdx2, TRIM31, and the NLRP3 inflammasome. TRIM31, an E3 ubiquitin ligase, is directly regulated by Cdx2, which modulates its expression at the transcriptional level (Jahan et al., 2022). TRIM31 plays a critical role in regulating NLRP3 protein levels by targeting it for ubiquitination and proteasomal degradation, thus acting as a negative regulator of inflammasome activation (H. Song et al., 2016).

Upon activation by specific danger signals, the NLRP3 inflammasome forms a multiprotein complex that facilitates the recruitment and autocatalytic cleavage of pro-caspase-1 into active caspase-1. This activation leads to the cleavage of GSDMD, releasing its N-terminal domain, which inserts into the plasma membrane to form pores initiating pyroptosis, a highly inflammatory form of programmed cell death critical for pathogen defense and inflammation (Dai et al., 2023).

Given the intricate interplay between Cdx2 and NLRP3, I hypothesized that Cdx2 plays a critical role in modulating inflammasome activity and GSDMD-mediated pyroptosis in intestinal epithelial cells. To test this, I assessed on the canonical pathway of GSDMD-mediated pyroptosis triggered by NLRP3 activation, comparing WT and *Cdx* mutant intestinal epithelial cells and observed distinct differences in GSDMD cleavage and subcellular distribution. These findings point to a role for Cdx2 in both inflammasome activation and GSDMD-dependent inflammatory cell death in the intestinal epithelium.

This study uncovered a strong link between *Cdx* deficiency and pyroptotic cell death, as evidenced by a marked increase in GSDMD cleavage and elevated cell death in *Cdx* mutant cells. Blocking the NLRP3 inflammasome led to a significant reduction in GSDMD cleavage, highlighting the role of *Cdx* in regulating NLRP3-driven pyroptosis. Additionally, subcellular fractionation and Western blot analyses confirmed that cleaved GSDMD relocates from the cytoplasm to the membrane in mutant cells, where it undergoes oligomerization leading to potential pore formation.

These results highlight the molecular mechanisms by which Cdx2 influences epithelial inflammation through its regulation of the TRIM31-NLRP3-GSDMD axis. Dysregulation of this pathway may contribute to the chronic inflammation observed in conditions such as IBD. Importantly, this work builds on evidence from Bulek et al., (2020), which demonstrated increased expression of epithelial-derived GSDMD in patients with IBD and suggested that GSDMD plays a pivotal role in driving tissue inflammation and damage in IBD, emphasizing its importance in disease pathogenesis. By linking Cdx2

regulation to GSDMD-mediated pyroptosis, the present study both supports Bulek et al.'s findings and also extends them by identifying Cdx2 function as a potential upstream regulator.

Cdx dependent regulation of GSDMD

Cdx2 has long been recognized for its essential role in the differentiation and homeostasis of intestinal epithelial cells, including playing a pivotal part in the maintenance of the epithelial barrier (Coskun et al., 2011). Specifically, Cdx2 regulates the expression of genes critical for intestinal epithelial differentiation, including solute transporters such as *SLC26A3* (Chatterjee et al., 2017). Moreover, Cdx2 modulates the expression of tight junction proteins and mucins, both of which are integral to preserving the intestinal barrier's integrity and defending the gut against harmful microbial invasion (Gao et al., 2009;Yamamoto et al., 2003). Disruption of *Cdx2* expression can compromise the epithelial barrier, thereby increasing susceptibility to inflammation and microbial dysbiosis (Coskun et al., 2011).

To investigate the role of Cdx2 and its effect on Gasdermin D (GSDMD) in intestinal epithelial cells, I developed a robust method for isolating viable epithelial cells via fluorescence-activated cell sorting (FACS). This ensured the purity of the epithelial cell population being analyzed, minimizing confounding effects from immune cell infiltrates. This is important as previous studies have shown that *Cdx2* deficiency is associated with increased intestinal immune cell recruitment, contributing to enhanced inflammation (Chewchuk et al., 2021; Jahan et al., 2022).

Through Western blot analysis of sorted epithelial cells, I found that *Cdx* mutant cells exhibited significantly higher levels of cleaved NT-GSDMD compared to WT controls. This suggests that disruption of *Cdx* in intestinal epithelial cells leads to increased GSDMD processing, consistent with the upregulation of NLRP3 in these cells (Jahan et al., 2022). Notably, a study demonstrated that cleaved

GSDMD also plays a non-pyroptotic role in driving mucus secretion, shaping the host-microbial interface (Zhang et al., 2022). Their findings highlight the non-pyroptotic functions of GSDMD, particularly its role in maintaining epithelial barrier integrity through mucus layer formation rather than inducing pyroptotic cell death.

Cdx2 has been shown to play a key role in regulating goblet cell differentiation and the production of mucins, such as MUC2, which are crucial for maintaining an effective intestinal barrier (Mesquita et al., 2003; Yamamoto et al., 2003). In the study by Zhang et al., (2022), immunofluorescence staining revealed a significant enrichment of GSDMD expression in *Muc2*-positive goblet cells, suggesting a potential link between Cdx2, GSDMD, and Muc2 in the regulation of mucus production. Additionally, our lab's research (Hryniuk et al., 2012) found that the loss of *Cdx2* led to a mis-patterning of goblet cells, as evidenced by Alcian blue staining. This mis-patterning may result in mucus with altered structural integrity or glycosylation, ultimately diminishing its efficacy despite increased production. Therefore, while cleaved GSDMD may promote mucus secretion, the overall quality and composition of the mucus could be impaired in *Cdx* mutant cells. To better understand the relationship between increased GSDMD activation and mucus production in *Cdx* mutant cells, future research could focus on analyzing the composition of mucus, specifically MUC2 levels and glycosylation patterns.

It's also possible that the Cdx-GSDMD pathway and the role of GSDMD in mucus production operate independently. The Cdx-GSDMD pathway is primarily associated with pore formation and cell death through pyroptosis, whereas Zhang et al.'s study focused on the non-pyroptotic function of GSDMD, where active GSDMD forms pores necessary for Ca^{2+} influx, which in turn activates and stabilizes scinderin for F-actin remodeling and subsequent mucin secretion via exocytosis in secretory granules. GSDMD may utilize a common membrane-targeting mechanism to form pores, but the outcome could differ depending on the physiological context. Further studies are necessary to assess both pyroptotic

and non-pyroptotic functions of GSDMD in the *Cdx* mutant background to clarify any potential balance between these activities and their impact on intestinal homeostasis.

NLRP3 activity is essential for *Cdx*-regulated GSDMD cleavage.

The cleavage of GSDMD is tightly regulated by inflammasomes NLRP3. The NLRP3 inflammasome also orchestrates the maturation of pro-IL-1β and pro-IL-18 into their bioactive forms while concurrently facilitating the proteolytic cleavage of GSDMD. Thus, GSDMD processing occurs in parallel with the activation and secretion of IL-1β and IL-18, providing a mechanistic link between inflammasome activation and pyroptosis.

This study demonstrated that *Cdx* mutant intestinal epithelial cells exhibit increased levels of NT-GSDMD, consistent with prior observations that NLRP3 activation is upregulated in these cells (Jahan et al., 2022). In agreement with this, treatment with CY-09, a specific NLRP3 inhibitor, significantly reduced NT-GSDMD levels in *Cdx* mutant cells compared to controls, indicating that GSDMD cleavage in these mutants is largely NLRP3-dependent. Interestingly, CY-09 has also shown promise in other contexts, such as inflammasome-driven pyroptosis in neuronal cells following cerebral ischemia/reperfusion injury (J. Zhou et al., 2023). Thus, the present findings contribute to a growing body of literature suggesting that CY-09 may be efficient in inhibiting NLRP3-mediated cell death across a variety of tissues, and points to the therapeutic potential of CY-09 in various disease contexts, including those where Cdx2 function is compromised.

Prior work demonstrated that TRIM31 levels were significantly reduced in UC samples compared to non-inflamed samples, concomitant with elevated inflammatory markers, including the NLRP3 product IL-1β (Jahan et al., 2022). Similarly, others showed that in a DSS-induced colitis model, the NLRP3 inflammasome acts as a central regulator of intestinal inflammation (Gao et al., 2019). Activation of

TLR4/NF-κB triggers NLRP3 inflammasome activation, promoting pyroptosis in intestinal epithelial cells (IECs) and driving chronic colitis in mice. These findings all suggest increased NLRP3 inflammasome activity in IBD. Other studies also provided evidence that a gain-of-function mutation of NLRP3 is associated with IBD (Schoultz et al., 2009; Verma et al., 2008). The intestinal epithelial barrier plays a critical role in maintaining selective permeability and preventing inflammatory damage. Key components, such as tight junction proteins (occludin, claudins, ZO proteins), are essential for the structural integrity of the epithelial layer, while the mucus layer, regulated by mucins like MUC2, serves as a first-line defense against pathogens (Kuo et al., 2022). Excessive NLRP3 activity disrupts these barriers by driving inflammation and epithelial damage. Therapeutically, CY-09 may restore barrier integrity in IBD patients by inhibiting NLRP3 activity, reducing IL-1β production, and preserving epithelial tight junctions and mucus layer function. Further research could investigate the broader implications of CY-09 in treating illnesses where NLRP3-driven pyroptosis contributes to barrier dysfunction, establishing it as a potential therapeutic target for reducing GSDMD-mediated damage in diverse organs.

The role of the NLRP3 inflammasome in IBD is far more complex than it initially appears. While a genetic study identified SNPs that likely reduce *Nlrp3* expression are linked to IBD, these findings contrast with other research showing that a gain-of-function mutation in NLRP3 is also associated with the disease (Schoultz et al., 2009; Verma et al., 2008). These seemingly contradictory results have sparked significant interest in investigating the role of the NLRP3 inflammasome in murine models of IBD. To address these inconsistencies, a study investigated the critical influence of environmental factors, such as intestinal microflora composition (Bauer et al., 2012). Their study revealed that the protective effect observed in $Nlrp3^{-/-}$ mice was associated with an increased presence of CD103+ lamina propria dendritic cells, which exhibit a tolerogenic phenotype under steady-state conditions. However, when $Nlrp3^{-/-}$ mice were cohoused with wild-type (WT) mice, the protective effect was lost, rendering

the $Nlrp3^{-/-}$ mice equally susceptible to colitis as their wild-type counterparts. Future studies are needed to further elucidate the roles of NLRP3 and GSDMD in IBD. For instance, investigating the regulation of GSDMD cleavage by NLRP3 in various IBD contexts could help determine whether NLRP3-driven pyroptosis plays a protective or harmful role in disease progression.

Caspase-1 activation is associated with pyroptotic cell death in *Cdx* mutant epithelial cells.

The observed increase in total cell death in *Cdx* mutant epithelial cells (~4.5%), as determined by 7-AAD staining, corresponds with heightened inflammatory signaling and cellular stress, which aligns with the expected consequences of NLRP3 inflammasome activation which is known to lead to epithelial damage and inflammatory cell death. Cell death can occur through multiple mechanisms, including apoptosis, pyroptosis, and necroptosis (Shen et al., 2023). The higher percentage of apoptosis assessed with the caspase 3/7 assay relative to total cell death, as measured by 7-AAD, may seem contradictory but can be explained by the basis of these assays and the biology of apoptosis. 7-AAD detects loss of membrane integrity by binding to DNA, indicating that the cell must be lysed for detection. In contrast, apoptosis is characterized by initial cell shrinkage without membrane rupture, meaning cells in the early stages of apoptosis may have caspase activity, but may not be detected by 7-AAD, as their membranes remain intact (Shen et al., 2023). This discrepancy could account for the observed difference between total cell death measured by 7-AAD and apoptosis detected by the caspase 3/7 assay.

The modest increase in apoptosis in *Cdx* mutant cells can be attributed to the potential crosstalk between apoptosis and pyroptosis within these cells. As demonstrated by Taabazuing et al., (2017), pyroptosis and apoptosis pathways engage in bidirectional interactions, suggesting that under conditions of cellular stress, such as inflammasome activation, both mechanisms can coexist and possibly exacerbate the overall cell death response. This interplay may help explain the increase in caspase 3/7-positive cells in

Cdx mutant cells, although further investigation is necessary to better understand apoptosis and pyroptosis in intestinal epithelial cells following *Cdx* loss.

To determine whether *Cdx* deletion induced pyroptosis, caspase-1 cleavage, which is a hallmark of inflammasome activation and required for GSDMD-dependent pore formation (Dai et al., 2023), was examined. The increased presence of cleaved caspase-1 in *Cdx* mutant cell supernatant supports pyroptosis as a major mechanism driving death in these cells.

The discovery of caspase-1 cleavage in *Cdx* mutant cells is also an exciting opportunity to investigate targeted therapeutics that may help target pyroptosis. Caspase-1 causes inflammatory cell death via GSDMD, coupled with processing and releases of the pro-inflammatory cytokines IL-1β and IL-18, which could further increase tissue damage. Blocking caspase-1 activity may interrupt this cascade by inhibiting GSDMD cleavage, minimizing the creation of disruptive membrane pores, and limiting inflammation-induced cell death, as well as maturation of IL-1β and IL-18. This dual effect, which reduces pyroptosis while also limiting cytokine maturation and release, has the potential to influence the course of inflammatory diseases. This approach is especially promising for IBD, which is characterized by chronic inflammation and epithelial damage; inhibiting pyroptosis may protect epithelial cells, reduce inflammation, and improve patient outcomes.

VX-765, a caspase-1 inhibitor that has shown promise in clinical trials, is considered a leading candidate for treating a range of diseases driven by caspase-1 activity. VX-765 effectively reduces IL-1β levels in macrophages, thereby alleviating disease severity in inflammation models such as rheumatoid arthritis and skin inflammation (Wannamaker et al., 2007). A more recent study (Jin et al., 2022) highlighted VX-765's therapeutic potential in reducing cognitive decline and neuronal damage following focal cortical infarction. The drug achieved this by decreasing β-amyloid deposition, inhibiting pyroptosis-

related proteins (such as caspase-1, NLRP3, ASC, gasdermin D, IL-1β, and IL-18), reducing markers of apoptosis (including Bax and cleaved caspase-3), and modulating inflammatory responses.

The use of caspase-1 inhibitors like VX-765 is part of a growing trend to target inflammasomes in diseases characterized by excessive pyroptosis and/or cytokine production. However, challenges remain, as inflammasomes play a crucial role in immune defense. Future therapies must balance reducing pathological inflammation while preserving the body's ability to fight infections. Further research should focus on enhancing these inhibitors to make them more disease-specific, particularly for disorders like IBD, to ensure their safety and efficacy.

NT-GSDMD forms membrane-associated oligomers suggesting the presence of pores in *Cdx* mutant intestinal epithelial cells.

One of the critical steps in pyroptosis is the translocation of cleaved NT-GSDMD from the cytoplasm to the plasma membrane, where it oligomerizes to form pores, leading to cell lysis. Consistent with loss of *Cdx* function leading to pyroptosis, the present study found that NT-GSDMD forms oligomers in the membrane. The complete absence of full-length GSDMD in the cytoplasmic fraction of unpurified populations of cells from *Cdx* mutants further suggests that GSDMD cleavage and translocation to the membrane occurs in all cell types present. In contrast, WT cell populations retained detectable levels of the full-length protein, reinforcing the conclusion that NT-GSDMD translocation from the cytoplasm to the membrane occurs in epithelial cells.

GSDMD oligomerization in *Cdx* mutant epithelial cells was also demonstrated through experiments using reducing and non-reducing conditions. In non-reducing conditions which preserved the oligomeric state, GSDMD complexes migrating above 180 kDa were observed in *Cdx* mutant cell isolates. The addition of a reducing agent resulted in GSDMD monomers from extracts from mutant cells, suggestive

of disassembled oligomers. Also notable was the observation that these high-molecular-weight complexes were significantly more prominent in the mutant cells compared to controls, where only minimal oligomerization was detected. Over the years, numerous strategies to inhibit GSDMD activity have been explored, with blocking pore formation being a key focus. A high-throughput screening approach with a fluorescent liposome leakage assay was used to investigate whether Disulfiram (DSF), a drug used to treat alcohol addiction, could function as a GSDMD inhibitor to prevent pyroptosis (Hu et al., 2020). Their findings revealed that DSF modifies cysteine residues (Cys191 in humans and Cys192 in mice) on GSDMD, rendering its N-terminal fragment (GSDMD-NT) unable to form pores. This discovery has sparked interest in the potential of DSF for treating inflammatory diseases such as ulcerative colitis (UC), even though earlier studies (Ou et al., 2021; Zhou et al., 2023) mainly investigated its effects on mechanisms independent of GSDMD.

While the translocation of cleaved NT-GSDMD to the plasma membrane is a critical event in pyroptosis, it does not always lead to cell lysis. Recent studies have uncovered mechanisms by which cells can repair or counteract membrane damage initiated by GSDMD pores, preventing cell lysis. One such mechanism involves the Endosomal Sorting Complex Required for Transport (ESCRT) machinery. Rühl et al., (2018) demonstrated that ESCRT-dependent membrane repair negatively regulates pyroptosis downstream of GSDMD activation by facilitating the excision of damaged membranes by forming complexes that recruit membrane vesicles, promoting membrane remodeling and sealing. This repair pathway is activated to counteract the damage caused by GSDMD-mediated pore formation, allowing cells to recover from membrane damage and preventing lysis.

Ceramide, a lipid involved in membrane dynamics, has also been shown to play a crucial role in regulating pyroptosis (Nozaki et al., 2022). Ceramide stabilizes membrane integrity after pore formation, acting as a line of defense against membrane damage during pyroptosis. Ceramide is produced through

the hydrolysis of sphingomyelin by acid sphingomyelinase (ASM), an enzyme that is activated during cellular stress or inflammation. ASM can increase ceramide levels at the site of membrane damage which promotes membrane repair by modulating lipid raft composition. By influencing these processes, ceramide helps mitigate the damage caused by pyroptosis, underscoring the versatility of cellular repair mechanisms.

Both ESCRT and ceramide-based pathways offer promising therapeutic targets. By promoting ESCRT-mediated repair, it may be possible to limit the extent of pyroptotic cell death and reduce tissue damage in diseases characterized by aberrant pyroptosis. Similarly, boosting ceramide production or ASM activity could provide a complementary approach to enhance membrane repair. Future studies are needed to explore the potential of targeting ESCRT and ceramide pathways in *Cdx* mutant intestinal epithelial cells to modulate pyroptosis and enhance membrane repair.

While processes such as ESCRT-mediated membrane repair are critical for limiting the degree of pyroptotic cell death, their role in cancer progression adds another layer of complexity. Chronic inflammation, as seen in IBD, creates a pro-carcinogenic environment by promoting DNA damage, immune dysregulation, and cellular stress, all of which contribute to colorectal cancer (CRC) development (Birch et al., 2022; Mak et al., 2020). Furthermore, others found that gut microbiota-mediated activation of GSDMD enhances colorectal carcinogenesis (Chen et al., 2024). Surprisingly, this activation is accompanied by the accumulation of ESCRT proteins on the plasma membranes of colorectal tumor cells which may aid in tumor cell survival by preventing pyroptosis. These findings suggest involvement of GSDMD and its related repair mechanisms in cancer, suggesting novel therapeutic options for certain cancers.

Future directions

My results have generated significant insight into the role of Cdx2 in regulating inflammasome activity and GSDMD-mediated pyroptosis in intestinal epithelial cells. These findings highlight the importance of Cdx2 in modulating inflammatory signaling and cellular responses to stress. Despite these advances, further investigation is needed to clarify the precise molecular mechanisms through which Cdx2 controls GSDMD-mediated pyroptotic cell death.

One limitation of this study is the inability to detect full-length GSDMD in sorted epithelial cells due to interference from the antibodies used in the sorting process. This challenge prevented a direct comparison of GSDMD levels between WT and *Cdx* mutant cells. However, in unsorted cells, full-length GSDMD is processed in *Cdx* mutant cells. Additionally, the significant increase in the active NT-GSDMD form in *Cdx* mutant sorted cells suggests that the loss of Cdx function enhances GSDMD cleavage, although further validation is required.

A potential solution for overcoming this limitation is the use of transgenic fluorescent reporter mice to refine epithelial cell isolation methods. These mice could be genetically engineered to express fluorescent proteins specifically in epithelial cells, enabling their direct identification and isolation via FACS without the need for antibody-based sorting. This method eliminates variability caused by antibody binding and reduces background from non-specific staining, ensuring more precise and consistent identification of epithelial cells. As a result, this method could enable the analysis of full-length GSDMD in sorted epithelial cells and potentially enhance the purity and reproducibility of isolated cell populations, thereby improving the reliability of downstream analyses. For example, transgenic fluorescent reporter mice with promoters such as *Lgr5* and *Musashi1* have been used to label intestinal stem cells (Maria Cambuli et al., 2013; Yan et al., 2012). Applying this technique to isolate epithelial cells for GSDMD analysis via Western blot could enable the assessment of both full-length and cleaved GSDMD, providing a means to validate the observations made in unsorted cells.

Another important limitation of this study is the inability to perform the subcellular fractionation analysis on sorted cells due to the insufficient number of cells available. One alternative approach for this would be to utilize electron microscopy to directly visualize the formation of pores at the membrane. This technique would provide a higher level of resolution and clarity in confirming the presence of these membrane pores, confirming the induction of pyroptosis in these cells.

One challenging aspect of GSDMD is that the formation of pores does not always lead to cell death. However, how could different cell types respond to these structurally similar GSDMD pores in different ways? One possible explanation is that the size and number of GSDMD pores could differ between cell types (Broz et al., 2020). For a cell to undergo lysis, the pores must reach a certain size (Dai et al., 2023). Therefore, assessing pore size in *Cdx* mutant cells could help support the occurrence of pyroptosis. Techniques such as electron microscopy and the Dextran leakage assay, which uses fluorescently labeled dextrans of different molecular sizes, can be employed. Smaller dextrans can pass through smaller pores, while larger dextrans can only traverse larger pores, providing a way to estimate the size of GSDMD pores.

An important future direction would be the development of an *in vitro* model to validate the findings described above. My initial attempt involved the use of colon organoids, chosen due to their simplicity and the absence of immune cells, which provides a "clean" system for studying epithelial-specific processes. However, I was unable to detect GSDMD or NLRP3 inflammasome components via Western blotting in this model, suggesting that colon organoids may not be suitable for this purpose. Small intestine organoids, on the other hand, might offer a viable alternative. The first step would involve verifying that these organoids express both GSDMD and components of the NLRP3 inflammasome at detectable levels. Once confirmed, deleting *Cdx* using 4-Hydroxytamoxifen could be used to generate *Cdx* mutant organoids. This would allow direct comparison between WT and mutant models within the same experimental framework.

After establishing a reliable small intestine organoid system, experiments from the mouse model could be systematically recreated. This would provide a critical layer of validation, confirming that the observed phenomena are reproducible in an independent in vitro model. Such a step would significantly strengthen the conclusions by demonstrating that the findings are not limited to a specific experimental context but are broadly applicable to intestinal epithelial systems and occur independent of immune (or other) cell types.

Establishing a reliable in vitro model, such as small intestine organoids, is a potential step toward validating the findings discussed earlier. However, it is equally important to consider the broader clinical implications of the research, particularly in the context of UC. One promising avenue for future investigation involves examining UC patients who display reduced levels of *Cdx2*, as low *Cdx2* expression has been linked to the presence of active UC (Coskun, 2014). This presents an intriguing opportunity to understand whether the loss of *Cdx2* in these patients may play a role in activating the NLRP3 inflammasome, potentially leading to increased levels of GSDMD and, consequently, pyroptosis in intestinal epithelial cells.

Given the relevance of this hypothesis to the clinical setting, an effective way to study the interaction between *Cdx2* loss and pyroptosis in UC would involve utilizing a specific mouse model for the disease. The DSS-induced colitis model, a well-established and widely used model of UC, would be particularly valuable in this regard (Okayasu et al., 1990). By comparing the levels of NT-GSDMD in *Cdx* mutant mice with and without DSS treatment, it may be possible to better understand how Cdx2 might influence the inflammatory processes in UC with respect to the activation of pyroptosis and its potential role in disease progression. This approach would not only strengthen our understanding of UC pathophysiology but also offer valuable information regarding the relationship between *Cdx2* expression, inflammasome activation, and pyropotic cell death in UC.

A comprehensive approach to investigating the interaction between Cdx2, NLRP3, and GSDMD, as well as confirming that epithelial cell damage primarily occurs through pyroptosis, is to knock out *GSDMD* using the CRISPR/Cas9 gene-editing tool. By creating a GSDMD knockout model, it becomes possible to directly assess the impact of GSDMD loss on pyroptotic pathways. Comparing the outcomes in WT and *Cdx* mutant cells, both with and without exposure to DSS, would provide critical insights into the role of GSDMD in mediating epithelial damage. This approach would not only help delineate the contributions of pyroptosis to cellular injury but could significantly advance our understanding of the molecular mechanisms driving inflammation and cell death in the context of *Cdx2* deficiency and inflammasome dysregulation.

Chronic inflammation in the gastrointestinal tract is a well-documented risk factor for the development of colorectal cancer (CRC) (Birch et al., 2022; Mak et al., 2020). Considering the significant involvement of the NLRP3 inflammasome in CRC (S. Li et al., 2025; Shao et al., 2020) and its role in driving inflammatory processes, it becomes crucial to explore whether inflammation induced by *Cdx* loss and subsequent NLRP3 activation play a role in CRC pathogenesis. If *Cdx*-induced inflammation contributes to tumor progression through the NLRP3 pathway, it would be insightful to examine if GSDMD activation in epithelial cells is also involved. A promising approach to test this hypothesis would involve using genetically modified mouse models that replicate both the loss of *Cdx* function and the progression of colorectal cancer. The AOM/DSS (azoxymethane/dextran sulfate sodium) model, commonly used for studying colitis-associated cancer (De Robertis et al., 2011), combines chemical carcinogenesis with DSS-induced colitis, closely mimicking the chronic inflammation seen in ulcerative colitis and its progression to CRC. By utilizing this model in *Cdx* mutant mice, it may be possible to assess the impact of *Cdx2*-dependent regulation of NLRP3 and subsequent GSDMD activation on CRC.

In addition to investigating the role of NLRP3 activation and GSDMD in CRC pathogenesis, it is crucial to underscore the prognostic potential of *CDX2* as a biomarker in CRC. Loss of *CDX2* expression has been strongly associated with poorer disease-free survival in stage II colon cancer patients (Dalerba et al., 2016). Furthermore, the absence of *CDX2* expression has been linked to various aspects of tumor progression, including increased stromal infiltration, which is often a hallmark of aggressive cancer behavior (Aasebø et al., 2020). In addition, restoring *CDX2* expression in CRC models has shown promising results, with studies indicating that re-expression of *CDX2* can reverse some of the aggressive phenotypic traits associated with CRC, leading to improved patient prognosis (Shigematsu et al., 2018; Tomasello et al., 2018). This highlights CDX2 not only as a potential prognostic marker but also as a therapeutic target in CRC.

Exploring the relationship between GSDMD activation and CDX2 status in CRC could open new avenues for therapeutic interventions. If *CDX2* loss promotes heightened inflammatory responses through the NLRP3-GSDMD pathway, this could suggest novel strategies for modulating inflammation-driven tumorigenesis. Understanding this interaction may lead to the development of targeted therapies that mitigate the inflammatory tumor microenvironment, thereby improving treatment outcomes and patient survival.

Summary

This study highlights the crucial role of Cdx2 in regulating inflammasome activity and GSDMD-mediated pyroptosis in intestinal epithelial cells. Our findings suggest that Cdx2 plays a significant role in controlling the activation of the NLRP3 inflammasome, which triggers GSDMD cleavage and leads to pyroptotic cell death. This process contributes to epithelial cell damage and likely contributes to loss of intestinal barrier function, both of which are key features of IBD and other chronic inflammatory conditions. Importantly, our results show that Cdx2 helps maintain intestinal health by balancing

inflammation and tissue repair. This opens up exciting possibilities for targeting the NLRP3 inflammasome and GSDMD as potential therapeutic strategies for treating IBD and similar diseases. By inhibiting NLRP3 or caspase-1, and promoting pathways that help repair the cell membrane (such as those involving ESCRT or ceramide), we could reduce the damaging effects of pyroptosis. This could help restore the integrity of the intestinal barrier, which is essential in preventing diseases like IBD from worsening. Additionally, improving barrier function could promote healing of the gut, reduce chronic inflammation, and lower the risk of complications such as colorectal cancer (CRC).

Going forward, it will be important to test these findings in intestinal epithelial cells that lack *Cdx2* in IBD and CRC models to better understand how its absence affects inflammasome activity and pyroptosis. Research into targeted therapies, like inhibitors of NLRP3 or GSDMD, could be key to improving outcomes for patients with IBD and CRC. Such therapies could help slow disease progression, reduce inflammation, and promote tissue repair, offering a new way to manage these challenging conditions.

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