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**Supplementary information**

# Proteases in intestinal health and disease

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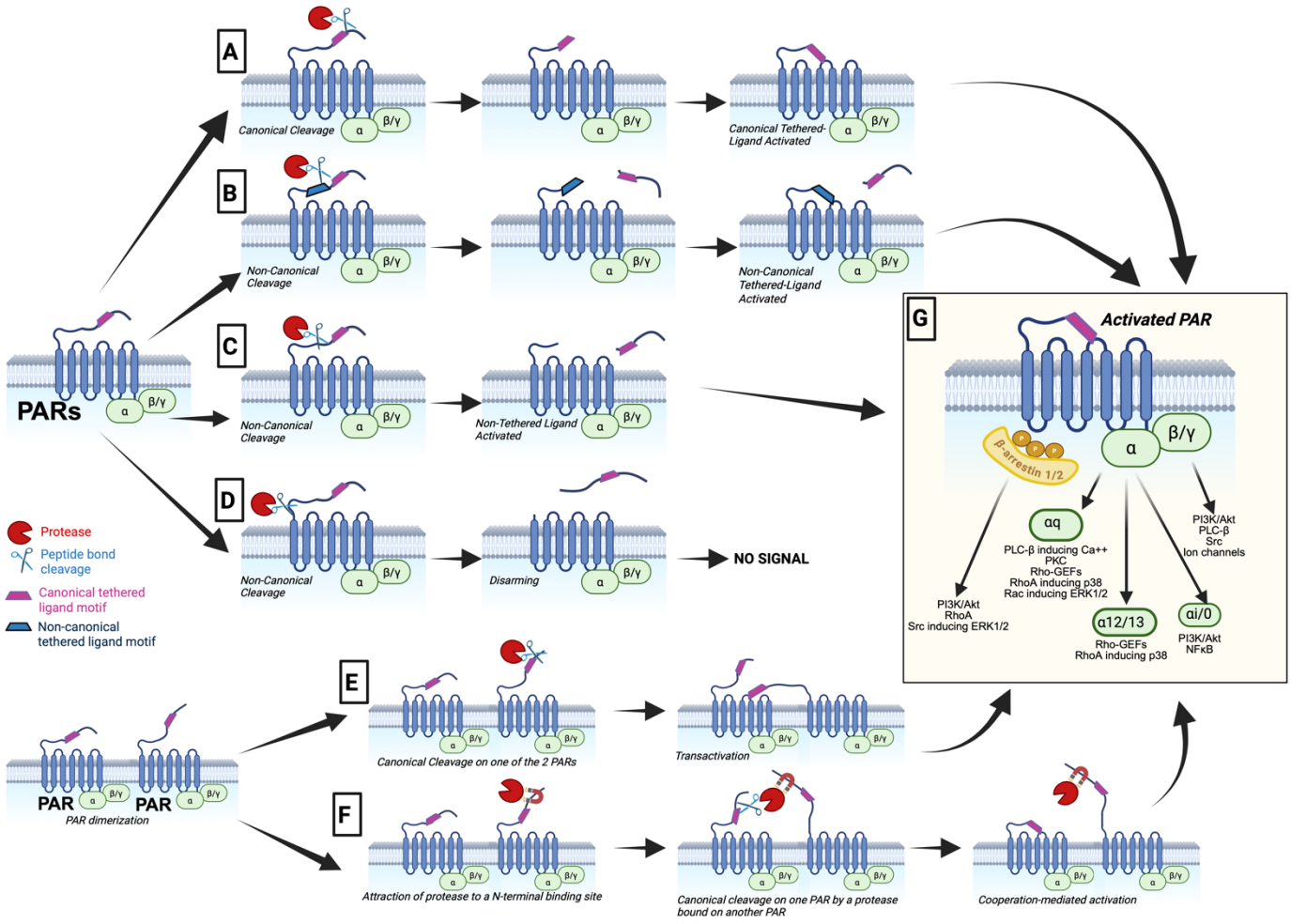


**Supplementary Figure 1:** Activation mechanisms of PARs (which are seven-transmembrane domain G protein-coupled receptors) by proteolytic cleavage of the extracellular N-terminal domain. Cleavage could occur on N-terminus at a canonical site (precisely and historically defined for each PAR), which reveals a new N-terminal domain that acts as a tethered ligand binding the second extracellular loop of the receptor and inducing intracellular signals. Thrombin activation of PAR1, or trypsin activation of PAR2 are typical examples of such activation (A). Cleavage could also occur at a non-canonical site and may unmask a different tethered ligand that could interact with domains of the receptor inducing biased signaling. Proteases such as elastase or MMP1 are typical examples of such mechanism of activation (B). Other proteases such as elastase can also cleave PARs and induce intracellular signals, without revealing a tethered ligand sequence (C). Proteases such as Cathepsin G or microbial proteases from *Pseudomonas aeruginosa* or *Porphyromonas gingivalis* can cleave PAR2 eliminating all possible tethered ligand mechanisms, thereby disarming receptor signaling by proteolytic cleavage. (D)

PARs may also function as dimers (homo- or hetero-dimers), where the cleavage of one of the PARs releases a ligand that binds and activates the adjacent PAR. This mechanism of activation known as transactivation (E) has been observed between PAR1, PAR2 and PAR4 by Cathepsin G for instance. Cooperation might also occur between PAR dimers, where one PAR binds efficiently a protease, which then has a facilitated access for cleavage to the adjacent PAR. This mechanism has been observed for thrombin which binds PAR3 in order for the adjacent PAR4 to be activated by thrombin proteolysis. This mechanism is known as a cooperation-mediated activation (F). Downstream signals from PAR activation (G) involves the dissociation of the GTP-bound Gα from the Gβγ subunits. Depending on the type of Gα bound protein, diverse signaling cascades include Phospholipase Cβ (PLCβ), which results in the production of inositol triphosphate and diacylglycerol, triggering calcium signals and Protein kinase C (PKC) activation, Ras homolog-guanine nucleotide exchanges (Rho-GEFs), Ras homolog family member A (RhoA), Rac-mediated MAP kinases ERK1/2, activation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) and nuclear factor-κB (NFκB). The Gβγ subunits dissociation involves PI3K/Akt, PLCβ and Src pathways, as well as coupling with ion channels. Finally, PARs may also signal through the mobilization of β-arrestin, a pathway independent of G-protein activation, which involves PI3K/Akt, RhoA and Src (ERK1/2) signals. Figure created using BioRender.

**Supplementary Box 1: Regulation of Secreted Proteases by Environmental Factors**

**pH Influence on Protease Activity**

Protease activity is highly pH-dependent, as the ionization state of amino acids in their active sites, such as aspartic acid, histidine, or serine, directly affects catalytic function. For example, the ionization of histidine in serine proteases catalytic triad is essential for catalysis. pH also influences substrate solubility, charge, and conformation, impacting binding and cleavage efficiency. Additionally, pH affects protease secretion and stability. In mast cells, the acidic pH (5.5) of secretory granules stabilizes tryptase by promoting ionic interactions with serglycin proteoglycans. Upon exocytosis into a neutral pH environment, these interactions weaken, leading to dissociation of the protease complex[1].

Acidic pericellular microenvironments, maintained by vacuolar H+-ATPases, can further sustain protease activity, such as that of secreted cathepsins[2].

### Redox Potential and Protease Function

Redox conditions critically regulate proteases with cysteine residues in their active sites, such as cathepsins and MMPs[2]. In reductive environments, disulfide bonds essential for structural integrity may break, impairing enzyme function. Conversely, oxidative conditions can lead to the formation of disulfide bonds, rendering cysteine proteases inactive. Reactive oxygen species (ROS) can modify residues like methionine, cysteine, and histidine, potentially inactivating proteases. Oxidative stress, often associated with inflammation and cancer, enhances proteolytic activity in the gut but may selectively influence specific proteases. Despite this, the direct link between redox conditions and gut protease activity remains poorly understood. Protease inhibitor can lead to structural changes in the inhibitors, such as modifications of disulphide bonds involved in the rigidity of the inhibitory loop (cystatins and serpins, notably), rendering them more susceptible to degradation by extracellular proteases.

### Role of Unconjugated Bilirubin in Protease Regulation

Bile acids also modulate protease activity, partly through microbial interactions. For instance, conjugated bilirubin in bile is converted to its unconjugated form by bacterial β-glucuronidase. Unconjugated bilirubin inhibits trypsin, illustrating a microbiome-mediated regulation of proteolysis[3]. In patients with irritable bowel syndrome (IBS), increased serine protease activity in fecal content correlates with reduced β-glucuronidase levels, suggesting an inverse regulatory relationship[4]. This highlights a potential microbiome-dependent mechanism for luminal protease modulation.

In summary, pH, redox potential, and bile acids collectively govern protease activity in the GI tract. These environmental factors affect enzyme stability, activity, and interactions, with implications for

health and disease states. Further research is needed to elucidate specific mechanisms, particularly regarding redox and microbiome-protease interactions.


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**Supplementary Table 1**

| PROTEASES CLASSIFICATION | | | |
|---|---|---|---|
| *Class* | *Catalytic Mechanism* | *Source* | *Examples* |
| **Serine Proteases** | A serine residue is present in the catalytic site and necessary for nucleophilic attack on peptide bonds | Host & Microbial | Trypsins, Chymotrypsins, Elastases, Thrombin, Subtilisin |
| **Cysteine Proteases** | A Cysteine-thiol group is necessary for nucleophilic attack on peptide bonds | Host & Microbial | Cathepsins, Caspases, Papain, Gingipains |
| **Aspartic Proteases** | Use two aspartic residues to activate a water molecule for nucleophilic attack on peptide bonds | Host & Viral | Pepsin, Renin, HIV protease |
| **Metalloproteinases** | Require metal ions (usually $Zn^{++}$) to activate water for peptide bond hydrolysis | Host & Microbial | MMPs, Thermolysin, LasB, GelE |
| **Threonine Proteases** | Use the hydroxyl group of a threonine as the nucleophile | Host | Proteasome, Ornithine acetyltransferase |
| **Glutamic Proteases** | Use glutamic acid residues to activate water for catalysis | Fungi | Scytalidoglutamic peptidase |

| | | | |
|---|---|---|---|
| **Others (mixed or unknown)** | Hybrid catalytic residues or unknown residues are required for catalysis | Microbial | Clostripain, microbial mucinases |

| **PROTEASE INHIBITORS CLASSIFICATION** | | | | |
|---|---|---|---|---|
| *Protein Family* | *Mechanism of action* | *Targeted Protease Class* | *Source* | *Examples* |
| **Serpin** | Suicide (irreversible) inhibition | Serine proteases | Host & microbial | α1-antitrypsin, Antithrombin, C1-inhibitor |
| **Kazal-type Inhibitors** | Competitive inhibition | | Host | SPINK1, PSTI |
| **WAP** (Whey Acidic Protein) | Reversible binding via disulfide rich WAP domain | | Host | Elafin, SLPI |
| **Staphostatin** | Reversible, substrate-mimicking | | Microbial | *Staphylococcus aureus* inhibitor |
| **Miropin** | Suicide inhibition | | Microbial | Gut commensal inhibitors |
| **Ecotin** | Variable | | Microbial | |
| **Cystatin** | Reversible, tight binding to the active site | Cysteine proteases (cathepsins, papain) | Host & Parasites | Cystatin C, Stefin, *Tryptosoma Leishmania* inhibitors |
| **Thyropin** | Active site blockade *via* thyroglobulin-like domain | | Host, Parasites & Microbial | Thyropin-1, Sgp3 |
| **TIMPs** | Reversible binding to Zn++ ion in active site | MMPs | Host & Microbial | TIMP-1 to -4 MS-60 |
| **α2-Macroglobulin** | Reversible (Trap) | Large Spectrum (serine, cysteine, metalloproteinase) | Host | |