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Review

The elegant complexity of mammalian ecto-5′-nucleotidase (CD73)

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Purinergic signaling is a fundamental mechanism used by all cells to control their internal activities and interact with the environment. A key component of the purinergic system, the enzyme ecto-5′-nucleotidase (CD73) catalyzes the last step in the extracellular metabolism of ATP to form adenosine. Efforts to harness the therapeutic potential of endogenous adenosine in cancer have culminated in the ongoing clinical development of multiple CD73-targeting antibodies and small-molecule inhibitors. However, recent studies are painting an increasingly complex picture of CD73 mRNA and protein regulation and function in cellular homeostasis, physiological adaptation, and disease development. This review discusses the latest conceptual and methodological advances that are helping to unravel the complexity of this important enzyme that was identified nearly 90 years ago.

Highlights

The ectoenzyme CD73, and its product adenosine, control cellular homeostasis and allostasis by integrating extracellular purinergic signaling with intracellular kinase activities and gene transcription.

CD73 is complex and coordinated at multiple levels, including transcriptional (by hypoxia), post-transcriptional (by production of an alternative splice isoform and circular mRNA), and post-translational (by *N*-glycosylation and shedding from the membrane to produce a soluble form).

An oxygen gradient patterns zonal expression of CD73 to regulate the long-term metabolic and immune stability of epithelial cells and tissues.

Multiple CD73 inhibitors are undergoing clinical development for cancer. Given the complex regulation and homeostatic functions of CD73 and adenosine, caution regarding the long-term safety of systemic inhibition of CD73 is warranted.

CD73 is an integral component of the purinergic system

Cells produce and consume ATP in a tightly regulated manner to ensure optimal organismal function. In addition to being used as fuel for essential activities within the cell, ATP is also released outside of the cell, where the sequential removal of its phosphate groups results in the formation of the nucleoside adenosine. Extracellular ATP and adenosine, together with associated synthetic and catabolic enzymes, receptors, and transporters, are part of the evolutionarily conserved purinergic system which links cellular metabolism to a myriad of other processes, including proliferation, differentiation, and cell death [1].

Extracellular ATP is rapidly metabolized by several enzymes collectively known as ectonucleotidases; these include members of the ectonucleoside triphosphate diphosphohydrolase (ENTPD) and ectonucleotide pyrophosphatase/phosphodiesterase (ENPP) protein families [2]. ENTPD1 (CD39) and ENPP1 generate adenosine monophosphate (AMP) from ATP (Figure 1). Subsequent hydrolysis of AMP to adenosine is primarily, but not exclusively, carried out by the enzyme 5′-nucleotidase (CD73) [3] (Figure 1). Extracellular adenosine activates four G protein-coupled adenosine receptors (ARs) which have different affinities for adenosine ($A_1R > A_{2A}R > A_3R >> A_{2B}R$) and activate numerous signaling pathways to control oxygen supply/demand, inflammation, and other activities, depending on the cell type and receptor expression pattern [4] (Figure 1). Adenosine can also be taken up via equilibrative nucleoside transporters (ENTs) and rephosphorylated to AMP inside the cell [5]. As the major enzymatic source of extracellular adenosine, CD73 is a key regulator of cellular homeostasis, stress responses, injury, and disease mechanisms across many tissues [6].

Currently, blocking the enzymatic activity of CD73 is regarded as an important avenue for cancer therapy because adenosine suppresses antitumor immunity [7]. Multiple CD73-targeting antibodies and small-molecule inhibitors are undergoing clinical testing (Figure 1). However, the long-term safety of systemic interventions blocking CD73 is an important consideration because adenosine is crucial for normal physiology, and loss-of-function mutations in the CD73-encoding gene (*NT5E*) cause a rare vascular disease in humans [8]. Moreover, studies over the past

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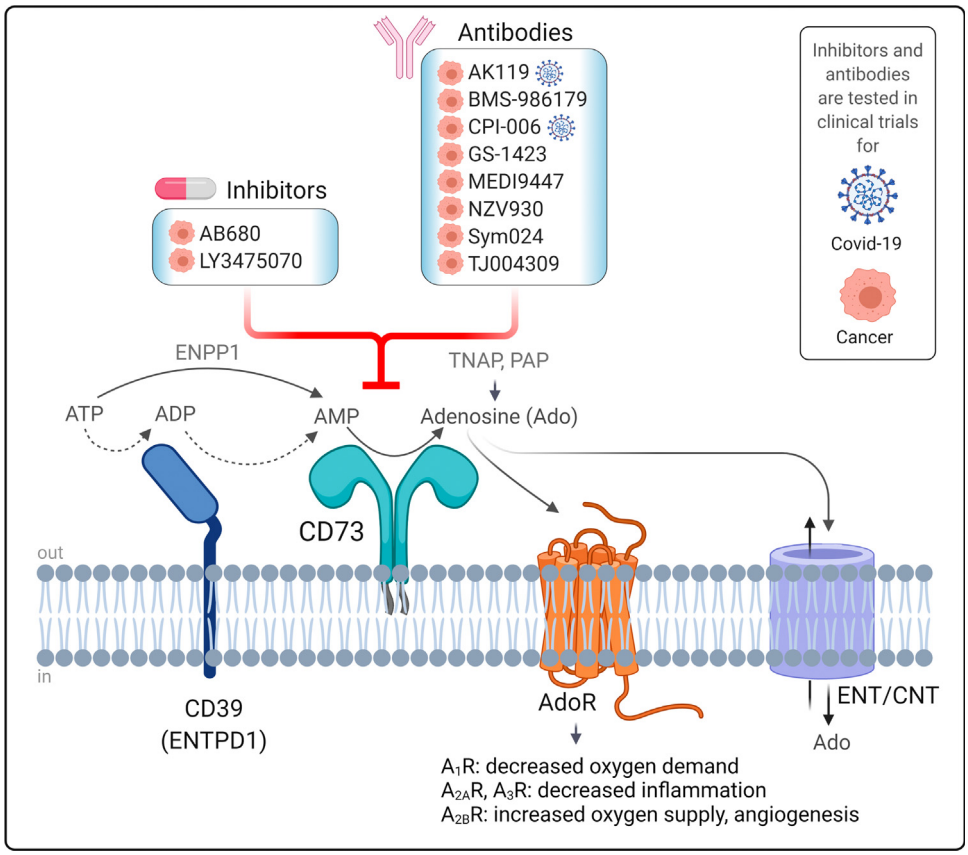

Figure 1. CD73 is an essential component of purinergic signaling and a disease target. CD73 is a ubiquitously expressed ectonucleotidase of the purine metabolism pathway. As a glycosylphosphatidylinositol (GPI)-anchored glycoprotein on the plasma membrane, CD73 works in tandem with ectonucleoside triphosphate diphosphohydrolase 1 (CD39) which breaks down ATP to form adenosine 5′-monophosphate (AMP) in a two-step process. Alternatively, AMP can be generated via direct conversion from ATP by the enzyme ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1). CD73 is the major enzyme that dephosphorylates AMP to generate extracellular adenosine (Ado), but this reaction can also be carried out by tissue non-specific alkaline phosphatase (TNAP) or prostatic acid phosphatase (PAP). CD73-generated adenosine directly exerts tissue-specific functions by binding to four different types of G protein-coupled adenosine receptors (AdoRs) which regulate oxygen supply/demand ratios, inflammation, and angiogenesis in a receptor-dependent manner. Extracellular adenosine is transported into the cytoplasm through equilibrative and concentrative nucleoside transporters (ENTs and CNTs). Given its role in inflammatory responses and tumor growth and metastasis, small-molecule inhibitors and monoclonal antibodies against CD73 are currently being tested in clinical trials for cancer immunotherapy and COVID-19 therapy.

2–4 years have illuminated significant biological complexity in human CD73 regulation and function which needs to be taken into account as the field moves forward. The purpose of this review is to place the latest discoveries on CD73 biology in a historical context and highlight CD73 functions that are important for normal cell biology and physiological homeostasis.

From 5′-NT to CD73: the evolution of CD73 biology and its links to human disease

CD73 is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein that functions as a dimer on the plasma membrane [9]. Discovered more than 80 years ago as 5′-nucleotidase (5′-NT), it received the designation 'cluster of differentiation 73' (CD73) immediately before the cloning of its cDNA in 1990 [3,6]. To distinguish it from functionally similar cytoplasmic enzymes [10], it is

also called ecto-5′-nucleotidase, abbreviated eN or eNT. Currently, 5′-NT, eN, eNT, and CD73 are all used to refer to the protein product (P21589; NP_002517) of the same gene, which is *NT5E* (Gene ID: 4907). The name CD73 is most commonly used in the recent literature (over the past 10–15 years) and coincides with a shift in focus on immune functions, especially in the context of cancer. However, CD73 is ubiquitously expressed and is involved in virtually every aspect of normal physiology and in many disease-associated processes [6]. Moreover, non-immune cells that normally express high levels of CD73, including fibroblasts and epithelial and endothelial cells, are epigenetically primed to elicit tissue-specific immune responses [11]. This is an important consideration, but one that is currently underappreciated.

CD73-generated adenosine controls cellular stress adaptation

Tissues with the highest level of *NT5E* expression include smooth muscle, the female reproductive system, liver, and gastrointestinal tract [6,12]. In addition to its abundance on epithelial and endothelial cells, CD73 is also active on neurons, glia, myocytes, and fibroblasts [6]. Based on many studies in whole-body *Nt5e*$^{-/-}$ mice, CD73 activity is important for maintaining tissue integrity, especially endothelial and epithelial barrier functions, and for facilitating recovery following hypoxia, ischemia/reperfusion, and inflammatory injury in the brain, heart, lung, kidney, liver, and digestive tissues [13,14]. A comprehensive description of the cell type- and tissue-specific functions of CD73 is beyond the scope of the current article, and we refer readers to previous reviews for more details [6,15]. Of note, there is existing evidence that CD73 also works non-enzymatically to regulate T cell receptor activation, immune–endothelial interactions, apoptosis, intracellular kinase signaling, and other cellular functions [6,15]. Nevertheless, most studies to date have attributed the physiological functions of CD73 to its ability to control the extracellular ATP–adenosine balance.

The basal release of ATP triggers activation of purinergic signaling to control diverse cellular processes, including ion transport, cell volume regulation, intercellular communication, blood flow, and neuronal activity [16]. Adaptability in this pathway is achieved through altered expression and activity of enzymes and receptors, and it is crucial for regaining homeostasis following exposure to stress, including mechanical, inflammatory, hypoxic, metabolic, and other types. For example, the mechanical stress-dependent release of ATP is particularly well documented across many cell types, such as erythrocytes [17], airway epithelial cells [18], vascular endothelial cells [19], astrocytes [20], and neurons [21]. Increased ATP release from stressed or dying cells represents a 'danger' proinflammatory signal, and is terminated upon ATP metabolism to the anti-inflammatory mediator adenosine to avoid excessive activation of tissue defense mechanisms [22,23].

Adenosine performs life-preserving functions inside and outside the cell

Adenosine has fascinated biologists for decades because it controls virtually every system in the body. It has been named a 'retaliatory metabolite' because it enables target cells to respond to stress and adjust their energy supply, thereby retaliating against external stimuli that would otherwise promote the excessive breakdown of ATP [24]. External stimuli can be physiological or stress-related, and vary by cell and tissue type (e.g., altered blood flow or tissue oxygenation, exposure to hormones, neurotransmitters, and inflammatory mediators) [25]. More recently, adenosine has been called a 'multi-signaling guardian angel' because it restores the oxygen supply-and-demand balance and exerts potent anti-inflammatory effects to guard against cellular damage [26]. Adenosine is taken up into the cell and rephosphorylated to replenish intracellular ATP stores, which is an important mechanism for purine salvage [27]. In addition, transport-dependent adenosine uptake and phosphorylation by adenosine kinase promote increased levels of AMP and activation of the master metabolic regulator, AMP-activated protein kinase

(AMPK) [28], which is important for cellular and tissue homeostasis. For example, mice with a targeted deletion of *Nt5e* in hepatocytes exhibit significant hypoactivation of AMPK and develop spontaneous hepatocyte degeneration and liver injury [29].

The four ubiquitously expressed metabotropic adenosine receptors and their G protein-coupled activities regulate cardiovascular, respiratory, and immune functions, metabolism, neurological activity, and gastrointestinal and liver biology [4]. Direct activation of adenosine receptors by small molecules with selective affinity for each receptor type represents an important avenue in drug development for cardiovascular diseases, pain, cancer, diabetes, liver disease, and other disorders [30]. Recently, mice that lack all four adenosine receptors were generated and were reported to have a significantly shorter lifespan [31]. The decline in survival began at 15 weeks of age, reaching 50% by the time the mice were 30 weeks old [31]. Although this mouse model reveals that baseline adenosine signaling via adenosine receptors collectively is crucial for long-term organismal viability, the mechanisms leading to shortened lifespan are unknown. Going forward, this mouse model will be a useful tool to address the role of adenosine receptor signaling not only in homeostasis but also in allostasis – the process by which regulatory systems adapt under stress to regain homeostasis [32]. Shifting away from whole-body to tissue-specific *Nt5e*$^{-/-}$ mice will provide additional clarity on where CD73 function fits with respect to homeostatic and allostatic adenosine receptor signaling across different tissues.

CD73-generated adenosine controls cancer progression

Persistent changes in the activation of purinergic signaling pathways can promote the development of diseases that are driven by metabolic perturbations and chronic inflammation, such as cancer [33]. Mechanical forces and other stress-related mechanisms are particularly relevant to growing tumors because they promote the release of ATP from cancer cells [34]. Pharmacologic or genetic ablation of CD73 in mice leads to decreased conversion of extracellular ATP to adenosine and promotes antitumor immunity [7]. The persistence of an immunosuppressive environment in the presence of active CD73 is largely due to increased A_{2A}R and, to a lesser extent, A_{2B}R activation on multiple immune cell types, including natural killer (NK) cells, and effector and regulatory T (Treg) cells. Specifically, A_{2A}R signaling inhibits effector T cell proliferation and cytotoxic function [35–37], enhances Treg expansion and immunosuppressive activity [38], and inhibits NK cell maturation and target cell killing [39,40]. Signaling via A_{2B}R facilitates expansion of myeloid-derived suppressor cells (MDSCs) [41], and CD73 activity on MDSCs inhibits T cells and NK cells [42]. Persistent hypoxia and inflammation within the tumor microenvironment boost immunosuppressive responses by elevating extracellular adenosine through modulation of adenosine-related genes [43,44]. Currently there is a strong emphasis on understanding the functional role of CD73 as an immune checkpoint modulator [45–48], as well as on testing whether CD73 inhibitors can provide benefit to cancer patients when used in combinatorial immunotherapy regimens [7].

Despite these efforts, we do not yet have a thorough understanding of all of the mechanisms by which CD73 controls tumor biology, especially in settings where its functions are tumor-suppressing. It is known that sustained expression of CD73 on epithelial cells is associated with favorable outcomes in endometrial and bladder cancer patients [49–51]. In endometrial cancer, epithelial CD73-generated adenosine is necessary for A_1R-dependent cortical actin polymerization and cell–cell adhesion, whereas CD73 loss is protumorigenic because it compromises epithelial barrier function [51]. Therefore, future studies need to reconcile these opposing, cell type-specific roles of CD73 in cancer. Particular care should be exercised to avoid over-emphasis of CD73 function on immune cells over other cell types, given its ubiquitous expression.

Genetic deficiency of CD73 alters kinase signaling and gene regulation in a rare human disease

The crucial role of CD73 in human biology is illustrated by the fact that loss-of-function mutations in *NT5E* cause a rare adult-onset vascular disease, calcification of joint and arteries (CALJA; OMIM: 211800) [52]. Also known as arterial calcification due to deficiency of CD73 (ACDC), the disease is characterized by painful calcifications that affect the lower and upper extremities [8,53,54] as a result of diminished CD73 activity on smooth muscle cells. The tissue-specific presentation of ACDC is in line with recent quantitative profiling studies that revealed highest *NT5E*/CD73 expression in human arteries, in a comparison of 32 normal tissues [55].

Mechanisms linking *NT5E* mutations to clinical presentations are not fully understood, partly because genetic mouse models of CD73 deficiency do not reflect the human phenotype [52]. To overcome this limitation, ACDC patient fibroblasts and iPSC-derived mesenchymal stromal cells (MSCs) have been used to study signaling mechanisms altered in the absence of functional CD73 [56,57]. These studies reveal that ACDC patient fibroblasts have dysregulated transcription factor Forkhead box O1 protein (FOXO1) activity [57]. Furthermore, ACDC patient MSCs display increased activation of AKT kinase, mechanistic target of rapamycin (mTOR), and the 70 kDa ribosomal protein S6 kinase (p70S6K) in the presence of osteogenic stimuli [56]. In the absence of functional CD73, there is increased phosphorylation of AKT, leading to FOXO1 activation, which in turn promotes expression of TNAP. Increased TNAP activity is a known factor in promoting ectopic calcification in ACDC [56]. Moreover, decreased levels of intracellular adenosine as a result of elevated activity of adenosine kinase, which phosphorylates adenosine to AMP, exacerbate vascular inflammation in mice via epigenetic reprogramming of histone methylation [58]. This mechanism was shown to be dependent on the uptake of extracellular adenosine, further supporting the hypothesis that CD73 provides a key link between extracellular purinergic signaling and gene regulation.

It will be important to assess additional stress response mechanisms in ACDC model systems because CD73 regulates stress recovery of bone marrow stromal cells [59]. Specifically, it was shown that depletion of CD73 from stromal cells impairs early hematopoietic cell regeneration following irradiation in mice. Understanding the detailed mechanisms behind these observations will be important in oncology settings because systemic use of CD73 inhibitors could affect the ability of cancer patients to recover from cytotoxic stress due to chemotherapy or radiation. As the natural history of ACDC becomes defined, patient-derived cellular tools to model aspects of the disease will enable further refinement of the cell-biological consequences of CD73 genetic inactivation or inhibition.

CD73 is zonally expressed on subsets of cells within epithelial tissues

Despite the noted species differences, animal models will continue to play an important role in advancing CD73 biology because we are also beginning to recognize the importance of zonal distribution of CD73 on subsets of cells within a given tissue, in particular the digestive epithelia (Figure 2). Advances in single-cell sequencing technologies are revealing how spatial dynamics may play a role in the physiological homeostatic functions of CD73, particularly in response to tissue oxygenation. Under homeostasis, CD73 defines populations of cells residing in low-oxygen areas, such as the villus tip enterocytes of the small intestine [60], the pericentral hepatocytes in the liver [61], and erythropoietin-producing interstitial cells of the kidney [62]. The zonal distribution of CD73 is consistent with earlier findings that its expression is strongly responsive to hypoxia because of the presence of hypoxia response element 1 (HIF-1) binding sites in the *NT5E* promoter [63]. This regulatory mechanism is similar between human and mouse. *Nt5e*$^{-/-}$ mice display vascular leakage in response to normobaric hypoxia in multiple tissues (lung, liver, gut, muscle, heart, kidney, brain) [13]. Furthermore, *Nt5e*$^{-/-}$ mice have increased susceptibility to cardiovascular, respiratory,

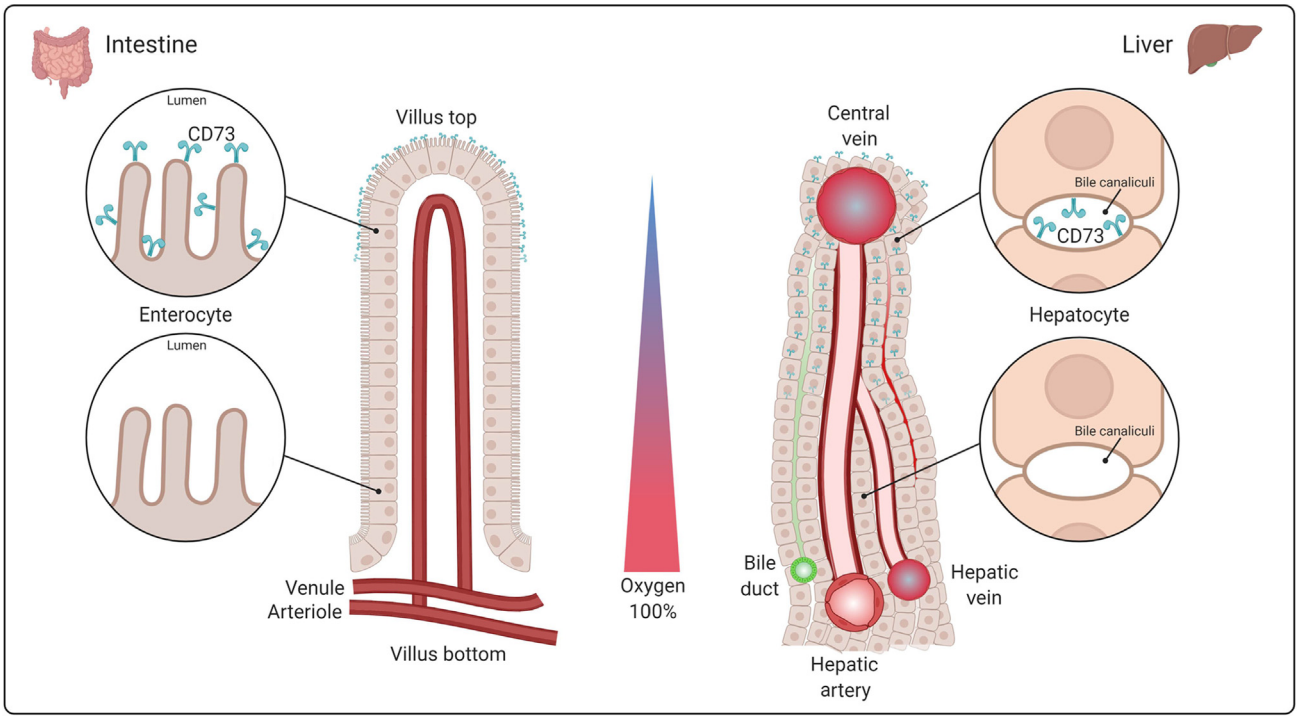

Figure 2. Zonal expression of CD73 supports tissue-specific homeostasis. A hypoxic environment induces CD73 expression on the apical membrane in digestive epithelia. In the intestinal epithelium CD73 is present on villus tip enterocytes, which are exposed to low-oxygen conditions. The villus tips face the intestinal lumen where anaerobic bacteria of the microbiome reside. Similarly, CD73 is zonally expressed on pericentral hepatocytes in the liver. In the hepatic lobule, periportal hepatocytes are located next to the portal triad (encompassing the hepatic artery, vein, and bile duct), while pericentral hepatocytes reside adjacent to the central vein. This arrangement follows the oxygen gradient: highly oxygenated blood enters the liver via the hepatic artery from the portal triad, mixes with deoxygenated blood through the sinusoids between hepatocytes, and is returned to the circulation via the central vein.

gastrointestinal (GI), and liver injury, in large part because they lack the adaptive mechanisms afforded by extracellular adenosine in response to hypoxia stress [13,64–67]. Therefore, the expression of CD73 in low-oxygen environments provides a physiological benefit that is suited to the architecture of the particular tissue type.

One reason for the preferential localization of CD73 in villus tip enterocytes is to counteract the ATP released by bacteria in the intestinal lumen and thereby control inappropriate immune activation by the host microbiome. Other potential functions may include the metabolism of cyclic dinucleotides to regulate host defense mechanisms at mucosal surfaces, and CD73 may also serve as a source of antimicrobial adenosine to prevent bacterial colonization and infection [68]. CD73 distribution on pericentral hepatocytes in the mammalian liver likely enables the cells to calibrate their metabolic activities under physiological low-oxygen conditions because genetic deletion of hepatocyte CD73 results in metabolic and inflammatory liver injury [29]. Because pericentral hepatocytes are involved in homeostatic renewal of the liver in response to Wnt ligands [69], it will be of interest to examine in the future whether CD73 modulates these pathways as part of liver mass maintenance.

Given these observations, it will be important to examine how purinergic intermediates in the gut, liver, and systemic circulation are affected by anti-CD73 interventions because this may affect the response of patients to, and effects of, immunotherapy. It was shown recently that the nucleoside

inosine significantly potentiates the anticancer effects of checkpoint inhibitors [70]. Inosine is derived metabolically from the gut microbiome [70] but can also be produced extracellularly from adenosine by cell surface-localized adenosine deaminase (ADA) [71]. It was observed that inosine translocates from the gut lumen to the systemic circulation where it activates T cell-specific $A_{2A}R$ signaling to promote antitumor T_H1 cell activation [70]. This raises a potential concern that coadministration of CD73 inhibitors (e.g., oleclumab) and checkpoint inhibitors (e.g., the PD-1 inhibitor durvalumab), as is being done in clinical trials, may reduce treatment efficacy by lowering inosine levels. Moreover, combining CD73 inhibitors with checkpoint inhibitors, which are known to cause acute hepatitis [72], may lower the threshold for liver injury and result in poor outcomes in the long-term [73], especially in patients with other predisposing factors for liver disease such as genetic makeup, age, and biological sex.

Sex and age are important variables in CD73 biology

An important question in moving forward is how biological sex will influence the safety and effectiveness of CD73-targeted cancer immunotherapies. Although there is some evidence based on meta-analyses [74] and multi-omic datasets [75] to support sex differences, this is still a highly debated issue and it remains an open question. At the basic science level, most *in vivo* studies on CD73 have been done only in male mice, or biological sex was not explicitly considered as a potential variable. However, an emerging concept in the field is that there are crucial hormonal influences, particularly estrogen-derived, in how males and females metabolize extracellular adenosine and cope with deficiency of CD73. Sex differences in adenosine signaling play a role in neuromodulation within the hippocampus, which has a high frequency of spontaneous transient adenosine events that regulate synaptic transmission, glia–neuron interactions, and other important functions [76]. Female *Nt5e*$^{-/-}$ mice have dramatically lower numbers of spontaneous transient adenosine events compared to wild-type (WT) mice, whereas male WT and *Nt5e*$^{-/-}$ mice have similar frequencies because of compensatory upregulation in TNAP in the latter [76]. Thus, female mice appear to be more reliant on CD73 for spontaneous adenosine transients, which is in line with earlier observations that CD73 expression and activity in the hippocampus are positively regulated by estrogen receptors (ER) α and β [77,78].

ERβ-mediated signaling affects CD73 biology in the GI tract via Tregs, which rely on CD73-generated adenosine for their immunosuppressive functions [79]. Specifically, estrogen mediates the differentiation of peripheral Tregs in an ERβ-dependent mechanism, and deletion of ERβ reduces the numbers of CD39/CD73-positive Tregs in female, but not in male, mice affected by chronic intestinal inflammation [80]. This finding has potential implications for the management of female versus male patients with inflammatory GI conditions because ERβ expression is selectively downregulated in the intestinal mucosa and circulating T cells of female Crohn's disease patients [80]. In the liver, hepatocyte-specific genetic deletion of CD73 leads to spontaneous injury characterized by metabolic dysfunction, hepatocyte swelling, and ballooning, steatosis, and inflammation in middle-aged male mice [29]. Female mice lacking hepatocyte CD73 are relatively protected, potentially via compensatory upregulation of *Entpd1* and all four adenosine receptors [29]. Sex-dependent differences in vulnerability versus resilience in coping with CD73 deficiency thus appear to be highly tissue-specific, and this will need to be resolved in future work.

Age and aging-related stress responses will be other important considerations for future studies, given recent findings that CD73 activity can be beneficial or harmful in atherosclerosis settings, depending on the age of the mice [81], and that CD73 expression levels change throughout the human lifespan [82].

CD73 expression and activity are regulated at multiple levels

Although many studies report that CD73 is altered in stress, chronic diseases, and cancer, few address the full spectrum of changes at the level of mRNA, protein expression and localization, or enzymatic activity (Figure 3). The specific mechanism by which CD73 is affected in stress and disease is an important consideration because upregulation at the mRNA level does not necessarily result in increased protein expression, and increased protein expression does not always correlate with increased enzymatic activity [83]. Moreover, the possibility that *NT5E* mRNA upregulation in cancer, as has been frequently reported, could act independently of CD73 activity has not been fully explored. Aside from HIF-1, several other transcription factors are known to induce CD73 expression, including SP1, SMAD, and c-Jun/AP-1 [84,85].

Recently, a novel tumor-promoting non-coding circular RNA with oncogenic activity, called circNT5E, was discovered in glioblastoma [86] and non-small cell lung cancer [87] (Figure 3). The circNT5E mRNA arises from the exon 3–9 region of *NT5E* through the activity of the double-stranded RNA-specific editase B2 (ADARB2). The protumorigenic activity of circNT5E is due to its ability to act as a sponge, or sink, for tumor-suppressor microRNAs (miRNAs), including miR-422a [86]. Adding to that complexity, miR-442a, miR-30a, miR-30b, miR-30a-5p, and miR-340 directly target and inhibit *NT5E* expression in head and neck, colorectal, gallbladder, glioma, lung, and pancreas cancer [88–90]. Other ways in which *NT5E* is dysregulated in cancer is via alternative splicing of exon 7 to produce a shorter enzymatically inactive intracellular protein isoform (CD73S), which acts as a dominant negative to the canonical form [91] (Figure 3). CD73S is a human-specific isoform and its exact functions remain to be determined. Unraveling these transcriptional mechanisms may open possibilities for selectivity in targeting the protumorigenic effects of *NT5E* without interfering with the normal enzymatic functions of CD73.

At the protein level, canonical CD73 undergoes several post-translational modifications (PTMs) that can significantly impact on its localization and activity, including cleavage from the membrane to form a soluble enzyme [92–94] (Figure 3). This is a key consideration in studies that involve tissue digestion because this removes the membrane-bound form of CD73, as was previously shown to occur immediately following hepatocyte isolation [83]. Another important consideration is that CD73 is *N*-glycosylated at four different residues (N53, N311, N333, and N403), and site-specific changes in the abundance and composition of glycans alter its subcellular localization and enzymatic activity [94]. Aside from human cirrhosis and liver cancer, presently little is known about how alternative splicing and glycosylation affect CD73 expression, localization, and activity in other chronic diseases and in different cancer types. Importantly, the transcriptional and post-translational CD73 regulation mechanisms operating under homeostatic conditions are not well defined, but it is likely that they exert significant effects on its function.

Development of CD73 inhibitors and other tools to support further research

Active efforts to block adenosine-producing CD73 activity for the therapeutic purposes of limiting cancer growth and metastasis include monoclonal antibodies and small-molecule inhibitors [95]. The initial proof-of-concept preclinical study using an inhibitory antibody against CD73 was performed 11 years ago [96], and at least five different anti-CD73 antibodies (BMS-986179, CPI-006, MEDI9447, NZV930, and TJ004309) and two small-molecule inhibitors (AB122 and LY3475070) are now in Phase I/II clinical trials [95]. Many similar agents are in early-stage discovery and preclinical development [95,97–102]. Somewhat surprisingly, some anti-CD73 antibodies are already being tested for coronavirus disease 2019 (COVID-19) therapy [103,104], despite evidence of clinical benefits of CD73 and adenosine in lung injury [105], and benefit of adenosine in pneumonia associated with COVID-19 [106]. CD73 activity on immune cells versus other types of cells (endothelial, epithelial) need to be carefully considered to advance safe and effective treatments for COVID-19.

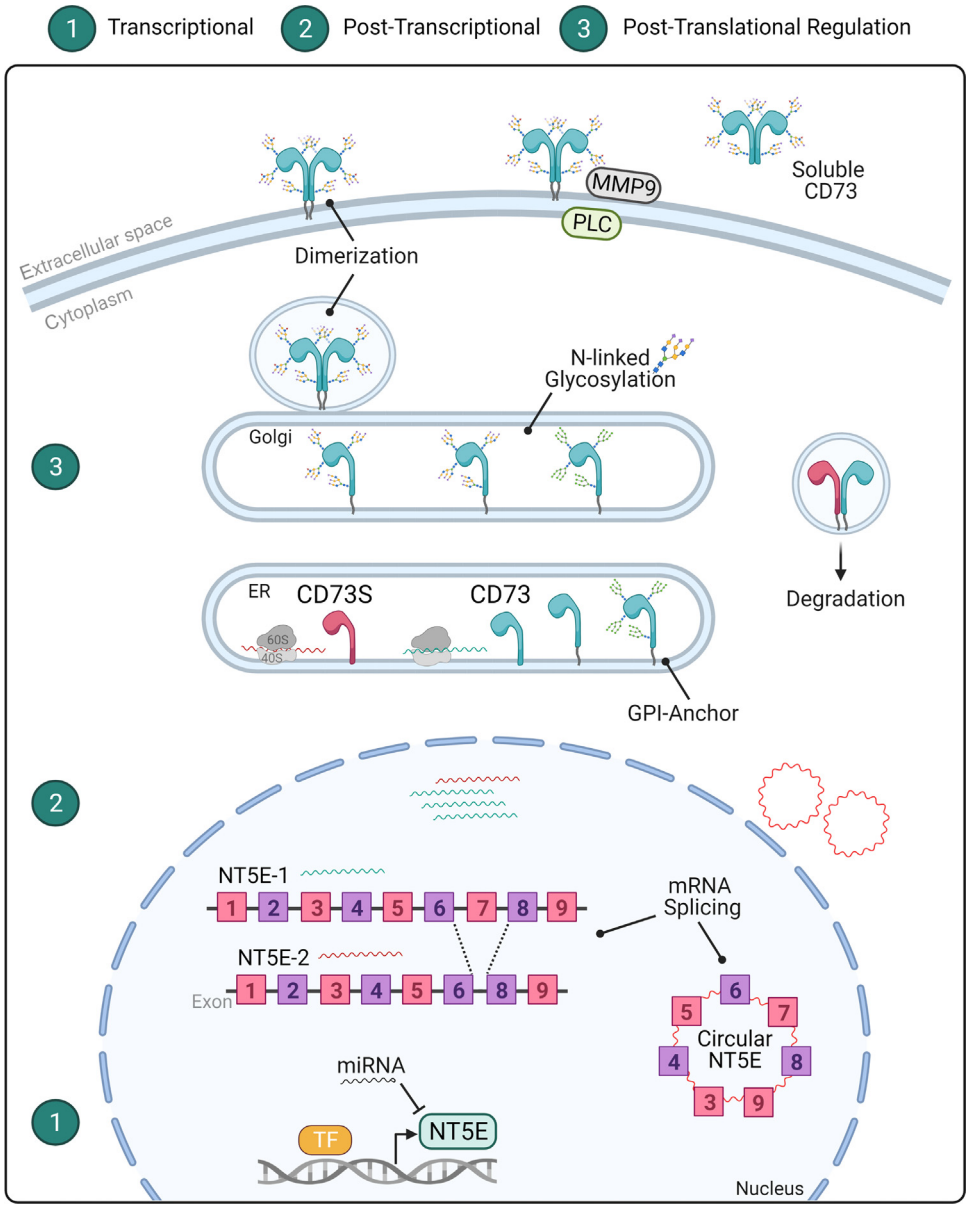

Figure 3. Molecular regulation of CD73. The important functions of CD73 across cell and tissue types warrant different levels of molecular regulation. (1) At the transcriptional level, the expression of the CD73-encoding gene *NT5E* is upregulated by transcription factors (TFs) such as HIF-1α, SP1, SMAD, and AP1, as well as by several microRNAs (miRNAs), as noted in the text. In the context of cancer, *NT5E* mRNA undergoes (2) post-transcriptional splicing to generate an alternative *NT5E-2* transcript or the circular RNA circNT5E. Subsequently, *NT5E-1* mRNA is translated into CD73 and *NT5E-2* mRNA into CD73S (for CD73 'short'). CD73S is an intracellular enzymatically inactive isoform that associates with, and promotes the proteasomal degradation of, canonical CD73 proteasomal degradation. The circNT5E transcript is oncogenic and is expressed in glioma and lung cancer. (3) At the post-translational level, CD73 protein is modified by the addition of a glycosylphosphatidylinositol (GPI)-anchor and by asparagine (*N*)-glycosylation in the endoplasmic reticulum (ER) and Golgi apparatus. The mature CD73 protein dimerizes and is transported to the plasma membrane facing the extracellular space. CD73 can be cleaved by phospholipase C (PLC) or matrix metallopeptidase 9 (MMP9) to generate a soluble form of the protein.

Elevating, rather than suppressing, the function of CD73 is likely to be beneficial in many situations where tissue inflammation needs to be reduced. To that end, bifunctional proteins were engineered by fusing the extracellular domains of CD39 and CD73 [107]. The fusion proteins exhibited high phosphohydrolysis activity toward extracellular ATP and antiplatelet activity *in vitro*, suggesting that they could potentially be developed to treat inflammatory diseases [107]. In addition, extracellular ATP release can be directly visualized in live animals using a newly developed optical sensor (ATPOS) [108], which represents another important methodological advance toward understanding the dynamics of the purinergic signaling components *in vivo*.

Radiolabeled antibodies [109] and fluorescent probes [110] are among the latest tools that have been developed to monitor CD73 distribution and regulation in various settings. An *Nt5e* reporter mouse was also generated, and it appears to be a useful tool for studying CD73 on multipotent stromal cells and sinusoidal endothelial cells [111]. The availability of multiple approaches to target, manipulate, and track CD73 will undoubtedly open new opportunities to understand its biology and regulation during physiological adaptation.

Concluding remarks

Decades of research breakthroughs on the release and metabolism of ATP to adenosine outside the cell have revealed crucial functions that are independent of essential metabolic activities occurring within the cell. Adenosine controls numerous homeostatic processes and stress-adaptation mechanisms, which would be rendered ineffective in the setting of chronic CD73 inhibition, an effort currently being undertaken in clinical research [7]. To successfully advance therapies around CD73, now is the time to take a step back and understand the fundamental biology behind this fascinating molecule (see Outstanding questions). Priorities for future work include the generation of additional human-specific tools to study CD73 regulation – such as iPSC-derived cells and tissue organoids [52]. These tools can help to resolve species-specific mechanisms such as alternative splicing [91], and help to streamline the process of translating preclinical discoveries to the clinic. It will be important for future studies to carefully consider the mechanisms by which CD73 expression and activity are altered in disease, such as mRNA expression and processing, protein expression, localization, and enzymatic activity, because these are often discordant under pathological conditions. The current anti-CD73 targeting strategies rely on the presence of the cell surface-expressed, enzymatically active form of CD73 but do not address alternative splice isoforms and PTM variants that can affect the localization and activity of the enzyme. Ideally, CD73 targeting in disease and cancer should be tailored to specific cell types to avoid untoward effects. The whole-body knockout mouse model has been instrumental in understanding CD73 function and for disease modeling [13] but, given the ubiquitous expression and complex interplay between CD73 on different cell types [6], it is crucial to move forward using tissue-specific knockout models, as has already been done in intestinal [112], kidney [113], and liver models [29]. All of these questions are addressable with the availability of new iPSC technologies, genetic mouse models, highly selective and potent inhibitors, and imaging probes, which are creating new opportunities to monitor, target, and manipulate CD73 (Figure 4). Future studies aimed at unraveling the biological complexity of CD73 regulation and functions will help to guide translational and clinical efforts for cancer and other human diseases.

Outstanding questions

Which aspects of CD73 regulation and function are conserved across species, and which are unique to humans?

What are the influences of age and biological sex, including hormones, on CD73 function, regulation, and roles in disease?

What is the significance of CD73 metabolic zonation, and how does CD73 mediate physiological adaptation in epithelial and non-epithelial tissues?

How are the transcriptional, post-transcriptional, and post-translational mechanisms integrated to control CD73 expression and activity in normal cells? How are these mechanisms altered during stress and in disease conditions?

What are the functions of the circNT5E mRNA in malignant neoplastic cells?

Which RNA-binding proteins control *NT5E* expression and splicing in homeostasis and stress?

How are major kinase signaling pathways (e.g., AKT, mTOR, AMPK) altered in the absence of functional CD73?

What are the non-enzymatic CD73 functions in different cell types, and how are they altered by CD73-targeting antibodies and small-molecule inhibitors?

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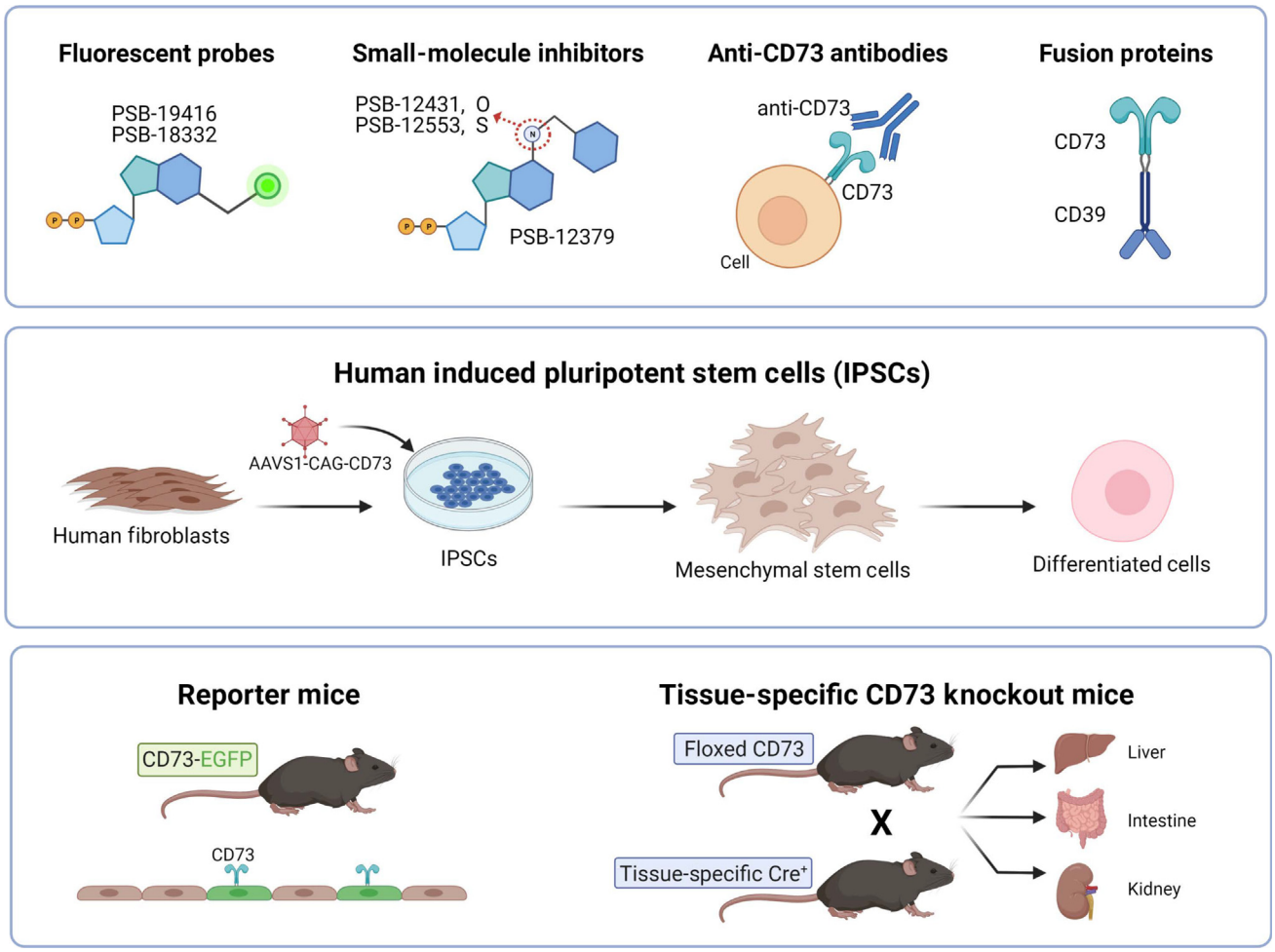

Figure 4. New tools to study CD73 regulation and function. The ubiquitous nature of CD73 and the purinergic signaling complexity conceal important tissue-specific mechanisms, which warrants development of new tools to study this ectoenzyme. (Top row) Fluorescent probes and small-molecule inhibitors were recently designed based on the lead structure of the most common CD73 inhibitor, adenosine 5′-(α,β-methylene)diphosphate (APCP). These newer probes exhibit higher potency while also enabling visualization and monitoring of CD73. In addition, studies demonstrating protumorigenic functions of CD73 led to the advent of new monoclonal antibodies that are being tested in clinical trials. By contrast, promoting CD73 activity may alleviate inflammation and platelet aggregation. To that end, a CD39–CD73 fusion protein was shown to sequentially hydrolyze proinflammatory ATP to anti-inflammatory adenosine. (Middle row) To interrogate relevant disease mechanisms, patient-derived iPSCs have become a robust model system. For example, fibroblasts derived from patients with a rare genetic mutation in *NT5E* can be reprogrammed to generate iPSCs. These, in turn, can be differentiated into affected cell types to study the pathological mechanisms of the rare disease ACDC (arterial calcification due to deficiency of CD73). (Bottom row) To elucidate the tissue-specific functions of proteins, reporter mouse lines and targeted gene deletion have been instrumental. A new reporter mouse line called CD73–EGFP enables cell lineage tracking and the identification of $CD73^{+}$ cells. Another useful model is the floxed CD73 mouse line, which enables targeted deletion of *Cd73* in specific tissues when mated with Cre recombinase mice. Specifically, deletion of *Cd73* in the liver, intestine, and kidney demonstrated tissue-specific protection under physiological and pathological conditions.

Declaration of interests

The authors declare no conflicts of interest.

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