Functions and mechanisms of protein disulfide isomerase family in cancer emergence

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Abstract
The endoplasmic reticulum (ER) is a multi-layered organelle that is essential for the synthesis, folding, and structural maturation of almost one-third of the cellular proteome. It houses several resident proteins for these functions including the 21 members of the protein disulfide isomerase (PDI) family. The signature of proteins belonging to this family is the presence of the thioredoxin domain which mediates the formation, and rearrangement of disulfide bonds of substrate proteins in the ER. This process is crucial not only for the proper folding of ER substrates but also for maintaining a balanced ER proteostasis. The inclusion of new PDI members with a wide variety of structural determinants, size and enzymatic activity has brought additional epitomes of how PDI functions. Notably, some of them do not carry the thioredoxin domain and others have roles outside the ER. This also reflects that PDIs may have specialized functions and their functions are not limited within the ER. Large-scale expression datasets of human clinical samples have identified that the expression of PDI members is elevated in pathophysiological states like cancer. Subsequent functional interrogations using structural, molecular, cellular, and animal models suggest that some PDI members support the survival, progression, and metastasis of several cancer types. Herein, we review recent research advances on PDIs, vis-à-vis their expression, functions, and molecular mechanisms in supporting cancer growth with special emphasis on the anterior gradient (AGR) subfamily. Last, we posit the relevance and therapeutic strategies in targeting the PDIs in cancer.

Introduction
Approximately one-third of the cellular proteome passes through the secretory pathway before they are secreted or translocated to their destined subcellular localizations. The endoplasmic reticulum (ER) constitutes the first compartment of this secretory pathway. It orchestrates the synthesis, folding, and structural maturation of many secreted and cell surface proteins. Initially, most polypeptides enter the ER unfolded. There, with the assistance of ER-resident proteins and chaperones, they fold and mature into proper tertiary or quaternary structures, before being transported to the second compartment i.e., the Golgi-apparatus for post-translational modifications such as glycosylation and lipidation [1].

Crucial to the functions of the ER are the ER-resident proteins in the ER lumen [2]. A major component of these ER-resident proteins includes the protein disulfide isomerases (PDIs), a class of multi-domains, multi-functional enzymes that belongs to the thioredoxin superfamily. The PDI family comprises several divergent proteins that can serve as molecular chaperones for protein synthesis and maturation [3]. As a folding catalyst, the PDI proteins are capable of preserving the native conformation and stability of other proteins via the formation, isomerization and rearrangement of disulfide (S–S) bonds. The formation of S–S bonds between two cysteine residues is highly conserved, and at least 30%...
of all eukaryotic proteins have at least one S–S bond, and ~80% of them are secreted or membrane proteins [4]. The essential roles of PDIs in oxidative protein folding are therefore conspicuous, especially the stability of the S–S bonds that can be perturbed by the reducing environments of most cellular compartments leading to protein misfolding. On the other hand, cellular functions are heavily dependent on the proper folding of proteins. To achieve proteome stability, the proteostasis network, that is comprised of conserved stress signaling pathways, involves in regulating the synthesis, folding, trafficking and degradation of proteins [5, 6]. Of note, ER proteostasis is likewise crucial because even the chronic expression of a single misfolded protein can result in the imbalance of proteostasis and impact multiple biochemical pathways [7–9]. These imbalances are associated with protein aggregation or proteinopathies which can lead to both loss- or gain-of-function diseases [10].

In this review, the structural properties and expression of PDIs in cancer are discussed. We describe existing and emerging roles as well as molecular mechanisms in supporting key biological processes in cancer emergence, with special emphasis on the anterior gradient (AGR) subfamily. In addition, the relevance and therapeutic strategies targeting the PDIs are highlighted.

### The composition and architecture of PDI family members

To date, twenty-one members of the PDI protein family have been identified, each having a different size, structure, tissue distribution and enzymatic activity (Table 1) [11]. A signature of the PDI family members is the presence of a catalytic thioredoxin fold (TRX), also known as the CXXC (Cys-X-X-Cys) motif, where X can be any amino acid. However, there are proteins in this family without the catalytic TRX or non-reactive TRX. Thus, a more accurate definition of the PDI family is that it (i) contains a non-thiol-reactive TRX motif with chaperone-like folding activities as well as (ii) having sequence and structural similarity to the TRX motif. The TRX motif contains two free thiol groups at each of the cysteine residues which mediate oxidoreductase activity that is important for shuffling the S–S bonds during protein maturation [12]. Most PDI family members have one to three TRX like-motif [13] and the most conserved TRX sequence for the PDI family is CGHC [14] (Fig. 1A).

### Table 1  The human PDI family members

| Gene name | Protein name | Aliases | Uniprot ID | Chromosome | PDB code |
|-----------|--------------|---------|------------|------------|----------|
| P4HB      | PDI          | PDIA1, PROHB, DSI, GIT, PDI, PO4HB, P4Hbeta | P07237 | 17q25.3 | 3bj5, 4ju5, 3uem, 6i7s, 4ekz, 4el1, 1x5c, 2bjx, 1bjx, 1mek, 2k18 |
| PDIA2     | PDIA2        | PDIA2, PDI, PDIR | Q13087 | 16p13.3 | None |
| PDIA3     | PDIA3        | PS8, ERP61, Erp57, Erp60, GRP57, PI-PLC, HsT17083 | P30101 | 15q15.3 | 2hl8, 2alb, 3f8u, 2dmm, 6eny |
| PDIA4     | PDIA4        | ERP70, ERP72 | P13667 | 7q36.1 | 3idv |
| PDIA5     | PDIA5        | PDIR, FLJ30401 | Q14554 | 3q21.1 | 46x |
| PDIA6     | PDIA6        | P5, Erp5 | Q15084 | 2p25.1 | 4ef0, 3vww, 4gwr, 3w8j, 1x5d |
| PDILT     | PDILT        | PDIA7 | Q8N807 | 16p12.3 | 5x7, 4nwy |
| ERP27     | ERP27        | FLJ32115, PDIA8 | Q96DN0 | 12p12.3 | 4f9z, 24c |
| ERP29     | ERP29        | Erp28, Erp31, PDI-DB, PDi9 | P30040 | 12q24.13 | None |
| ERP44     | ERP44        | KIAA0573, PDIA10 | Q98526 | 9q31.1 | None |
| TMX1      | TMX1         | TMX, PDI A11 | Q9H3N1 | 14q22.1 | 1x5e |
| TMX2      | TMX2         | PDIA12 | Q9Y320 | 11q12.1 | 2d0j |
| TMX3      | TMX3         | FLJ20793, KIAA1830, PDIA13 | Q96J7 | 18q22.1 | None |
| TMX4      | TMX4         | DJ971N18.2, KIAA1162, PDIA14 | Q9H1E5 | 20p12.3 | None |
| TXNDC5    | TXNDC5       | MGC3178, FLJ21353, FLJ90810, EndoPDI, Hcc-2, Erp46, PDIA15 | Q8NB59 | 6p24.3 | 3wgx, 3wge, 3uvt, 3wgd, 3uj1, 2diz |
| TXNDC12   | TXNDC12      | TLP19, ERP18, ERP19, hAG-1, AGR1, PDIA16 | Q95881 | 1p32.3 | 1sen, 2kv8 |
| AGR2      | AGR2         | XAG-2, HAG-2, AGR2, PDIA17 | Q95994 | 7p21.1 | 2ins, 2lnt |
| AGR3      | AGR3         | HAG3, HAG-3, BCMP11, PDIA18 | Q8TD06 | 7p21.1 | 3ph9 |
| DNAJC10   | DNAJC10      | Erdj5, PDIA19 | Q8IXB1 | 2q23.2 | None |
| CASQ1     | CASQ1        | PDIB1 | P31415 | 1q23.2 | 5crg, 5crq, 5crn, 3uom, 5cre |
| CASQ2     | CASQ2        | PDIB2 | Q14958 | 1p13.1 | 60nv, 60nn, 2vaf |
bonds [15]. PDIA1 constitutes ~80% of total ER proteins and was also the first PDI to be identified as a catalyst for protein folding [16, 17]. Structurally, PDIA1 harbors four distinct domains i.e., a, a', b, b' [16, 18, 19] (Fig. 2). Other notable structural determinant includes the acidic C-terminal and x-linker (connecting a' and b'-domains). The TRX-like domain is typically categorized as harboring the a-type (catalytic) and b-type (non-catalytic) domains, whereby the a' and b' symbols are often used to indicate the positions of such domains in the polypeptide. The a-type domain contains a thiol group responsible for mediating S–S bond formation, while the b-type domains act as spacers for protein recruitment [20]. The x-linker is a 19-amino acid residue located in between the a' and b' domains that are deemed to cushion the substrate-binding on the b' domain [21, 22].

Other members of the PDI family differ substantially in terms of the a/b-domains configurations and other important motifs (Fig. 1A). Phylogenetic analysis that is based on sequence similarities of PDI reveals the existence of multiple subfamilies, markedly the AGR (TXNDC12, AGR2, AGR3), TMX (TMX1-4) and CASQ (CASQ1, CASQ2) subfamilies (Fig. 1B). The AGR subfamily and some thioredoxin-related transmembrane proteins such as the TMX protein subfamilies only possess the catalytic a-type domain, but not the b-type domain [22, 23]. Another notable feature of PDI proteins is the presence of an ER-retention motif at the uppermost C-terminus end. The founding PDIA1 protein contains a highly acidic tetrapeptide ER-retention motif KDEL (Lys-Asp-Glu-Leu) and is considered a canonical motif for ER retention. This motif is recognized by KDEL receptors in a post-ER compartment and such binding triggers retrieval back to the ER, thus ensuring that the proteins harboring this motif stay in the ER. However, there are variations of this motif (e.g., KTEL, EDEL, QSEL) within the PDI family suggesting that some PDIs have different tolerances in binding affinity to KDEL receptors and thus may escape the ER or are secreted into extracellular space [24]. The CASQ1, CASQ2 ERP27, and ERP29 proteins do not carry the TRX motif and contain only the b-type domains suggesting that they are incapable of mediating S–S bonds. Interestingly, the CASQ subfamily proteins are the only PDI proteins missing the ER-retention motif. Another interesting subset of the PDI family is the more recently discovered transmembrane domain-containing TMX subfamily of proteins (TMX1-4) suggests possible protein localization in the ER membrane or other membranous organelle. Additionally, phylogenetic studies revealed that members of PDI and...
Rho guanine-dissociation inhibitors (Rho-GDI) have a syntenic linkage and were co-regulated by the same cis-regulatory elements, suggesting that PDI/RhoGDI pairs have a common ancestor and functional linkages [25].

Core functions of PDI members
The core function of PDI-like protein members is to facilitate the formation of disulfide bonds for secretory and membrane proteins entering the SP, particularly in the ER [26–29]. This ensures that the client proteins are in a proper stabilization and maturation state prior to ER exit. The S–S bond formation occurs primarily in the ER under an oxidative environment, in which they are formed through oxidizing thiol-reactive cysteine and then isomerizing them to attain the right conformation.

To understand the formation of disulfide bonds of proteins travelling along with the SP, it is worth reviewing the enzymatic activity of the founding member PDIA1. In the process of native protein folding, PDIA1 undergoes a redox reaction. The oxireduction cycle of PDIA1 alters the conformation to enable correct protein folding. When PDIA1 is in an oxidized state, PDIA1 mediates the enzymatic catalysis of dithiols to disulfides by pairing cysteines in the active site with reduced nascent polypeptides or substrates to form a disulfide bond [30, 31]. The reduced cysteine thiols in the substrate bind to the CGHC disulfide to produce a PDI-protein complex. The complex interacts with a second reduced thiol from the substrate, resulting in an oxidized and stable native protein. Simultaneously, the disulfide in the active site of PDI is reduced to the dithiol state [20]. While PDIA1 is in a reduced state, it catalyzes the breakage of disulfide bonds of the oxidized protein substrates and reduced it to dithiol. To complete the catalytic cycle of PDIA1, the PDIA1 protein must be reoxidized by electron transfer, for example by an electron transport machinery involving an enzyme ER oxidoreductin 1 (Ero1) [32]. Isomerization, on the other hand, occurs during PDIA1 being in a reduced state. The cysteine closest to the N-terminus active site initiates the intramolecular rearrangement of S–S bonds [33]. Client proteins enriched in cysteines are predisposed to error, especially with regard to intramolecular disulfide bonds, hence isomerization is required to convert incorrectly formed disulfides to their native conformation. Isomerization can be thought of a series of reduction and oxidation cycles [15].

PDIs have also been proposed to act as molecular chaperones [34]. A fundamental characteristic of a molecular chaperone is the ability to bind to a partially or completely disordered client protein. When chaperones bind
to the substrates, the protein substrates are not in their native functional conformation. Non-native states exist in all proteins in the cell at some point during their life cycle. For example, unfolded polypeptides that have only been translated may bind to chaperones before reaching their native conformation. Chaperones are crucial in maintaining proteostasis. This ability of a molecular chaperone to interact with the misfolded protein is primarily to protect their client protein from aggregation and is often referred to as ‘holdase’ activity. Whereas a process of a chaperone assisting a protein in folding back to its native state is called ‘foldase’ activity. Interestingly, PDIs are shown to be capable of distinguishing between native, unfolded or misfolded proteins through hydrophobic interactions [35]. Misfolded proteins that are lacking disulfide bonds can also bind to PDIs to prevent the aggregation of damaged proteins, suggesting their roles in the ER goes beyond their role in disulfide bond formation [36]. For PDIA1, it has been proposed that the molecular chaperone activities are independent of its redox activity [37, 38]. The C-terminus and α-domain were shown to be crucial for its chaperone activity. Truncation of the C-terminus and α-domain results in the loss of the chaperone function, however, it is not known whether this applies to all PDI family members [39, 40].

Since there are multiple PDI family members, their functions may be overlapped. It has been shown that PDI family members may have at least one function as described here, whether it be as oxidases, reductases, isomerases, or molecular chaperones. The differences in structural determinants especially concerning their catalytic thioredoxin fold, hydrophobic pockets, and ER retention motif result in their functions being specialized depending on cellular settings. PDI proteins are therefore crucial in ER proteostasis, which aids in the maintenance of several important cellular functions like gluconeogenesis, calcium storage, organelle biogenesis, and lipogenesis. As a result of its various influences on cellular stress, disruption of ER proteostasis frequently leads to the development of multiple disease states.

**Expression of PDI members in cancer**

Several PDIs are frequently overexpressed in cancers despite being one of the most abundant cellular proteins in the ER [41]. Assessment of PDI proteins expression using published microarray datasets revealed that PDIA1 is significantly upregulated in the brain and CNS cancers, lymphoma, kidney, ovarian, lung and male germ tumors. Analysis of cytosolic and cell surface proteomes derived from various cancers also yielded similar results [42]. Likewise, other PDI members namely PDIA3, PDIA4, and PDIA6 are also highly expressed in numerous cancer types including breast, thyroid, rectal, gastric and liver cancers [43–45]. Additionally, we analyzed publicly available RNA-Seq data of all PDI members that could give the overall picture of their expression in the cancer landscape. Integration and comparative analysis of TCGA (cancer tissues) and GTEx (normal tissues) RNAseq data showed almost similar trends to previous data in that PDIA1 showed a high level of expression in both normal and cancer tissues suggesting that PDIA1 is a universal catalyst of disulfide bond formation (Fig. 3). The expression of PDIA3, PDIA4, PDIA6, ERP29, and TXNDC5 have also upregulated in most cancer types. On the other hand, PDILT, CASQ1, and CASQ2 proteins are expressed at low levels in most tissues and their expression seems not to be differentially expressed in normal and cancer tissues. Perhaps, the most differentially expressed protein is AGR2 which is upregulated in lung, pancreas, stomach, breast, prostate, and colorectal cancer tissues compared to their normal tissues. It is important to note that these analyses are based on mRNA expression and that proteins can undergo various post-translational modifications (PTMs) that could alter their expression. Current database and proteomic technologies do allow us to comprehensively scrutinize the expression of each PDI. There are several proteomics studies, however, that identified PDI proteins as one of the upregulated proteins in cancer settings. For example, the mass spectrometry-based analysis identified an increased level of PDIA1 in prostate [46], breast [47], and brain [48] tumors. The upregulation of PDIA3 protein expression in colorectal, liver, brain and clear cell ovarian cancer was also revealed by high-throughput OMICS platforms [49–52]. The AGR2 protein was found to be upregulated in esophageal adenocarcinoma as shown by quantitative shotgun proteomics and immunohistochemistry [53].

**Post-translational modifications of PDIs**

It has been well-established that the structure, expression, and function of a protein can be altered via PTMs. Therefore, we discuss briefly the PTMs among PDI members that could expand our understanding of their roles. In a recent review, it has been shown that S-nitrosylation, carbonylation and S-glutathionylation can impair the functions of PDIs [54]. These PTMs are aberrant and irreversible, and they are the outcome of cellular nitrosative or oxidative stress, where high levels of reactive nitrogen species (RNS), hydrogen peroxide and reactive oxygen species (ROS) accumulate as modifying agents. Among these PTMs, S-nitrosylation of PDI has been demonstrated to inhibit the chaperone and isomerase activity of PDIs, which have been observed in neurodegenerative diseases [55, 56]. Whereas, S-glutathionylation, the formation of a disulfide bond between glutathione and a cysteine residue of another protein has been attributed to
protein misfolding and enhancement of the UPR and has been associated with cancer [57, 58].

Our investigation of the PhosphoSitePlus repository (www.phosphosite.org), on the other hand, revealed that all but one member (ERp27) of the PDI family can undergo extensive native PTMs, encompassing phosphorylation (p), acetylation (Ac), ubiquitylation (Ub) and glycosylation (G), methylation (M) and succinylation (Sc) [59]. To elaborate further, we found that human PDIA1 can accommodate up to 65 PTM sites i.e., Ac (12), G (1), M (7), p (19), Sc (9) and Ub (17). Similar to the other 19 PDI family members, most of these PTMs were identified with large-scale OMICS approaches, notably by mass spectrometry analysis. Of these 65 sites, only 3
sites have been investigated for their biological functions i.e., Thr331p, Ser357p and Ser427p by Yu et al., where they discovered that the phosphorylation of Ser331 and Ser427 affected the conformation and activity of PDIA1 minimally [60]. In contrast, when Ser357 becomes phosphorylated by the secretory pathway kinase Fam20C, PDIA1 adopted an open conformation, which converts PDIA1 from a “foldase” into a “holdase” for preventing protein misfolding in the ER. Besides, the phosphorylation of Ser357 allows PDIA1 to interact with the luminal domain of IRE1α, a UPR signal transducer to attenuate excessive IRE1α activity. Likewise, 83 PTMs have been reported for PDIA3 in PhosphoSitePlus, including Ac (13), G (4), M (8), p (36), Sc (7) and Ub (15). While it was documented earlier that Lyn, a protein tyrosine kinase of the Src family is primarily responsible for the phosphorylation of tyrosine residues Tyr445, Tyr454 and Tyr467 in PDIA3, little is known about the biological implications of these modifications [61]. PDIA3 is well-known as an essential component for MHC Class I peptide loading complex which presents antigen to CD8+ cytotoxic T cells to enable tumor antigen recognition and anti-tumor immune responses, hence it is vital for cancer immunotherapy [62]. We, therefore, articulate that further functional characterization of the various PTMs located in the PDI family is of critical importance.

**Functions and mechanisms of PDI members in cancer**

The high level of expression of PDIs in cancer has been shown to be associated with cancer progression, metastasis and invasion [63, 64]. The mechanisms by which PDIs promote tumour growth and metastasis has been linked with their ability to suppress apoptosis [48, 65, 66]. For example, ERp29 which lacks the catalytic a-type domain has been shown to downregulate eLF2α which in turn upregulates Hsp27, resulting in the inhibition of apoptosis in breast cancer cells. Besides, ERp29 may also promote cancer invasion and metastasis by regulating epithelial-mesenchymal transition (EMT) [63]. Inhibition of PDI activity by the PDI inhibitor, bactracin increased apoptosis in cancer cells and more evidently in response to ER stress [67]. Similarly, inhibition of PDIA1 activity by PDI inhibitor PACMA 31 reduced the growth of the mouse xenograft model of human OVCAR-8 ovarian cancer [68]. In line with this, siRNA-mediated knockdown of PDI demonstrated caspase-dependent apoptosis in the MCF-7 breast cancer cell line [69]. Meanwhile, overexpression of cytosolic PDIA1 has been shown to inhibit cell death after an apoptotic stimulus [70]. It was also shown that PDIA1 has non-canonical function in supporting ferroptosis in breast cancer cells and induced accumulation of lipid ROS [71]. In lung cancer cells, PDIA4 and PDIA6 were upregulated in response to cisplatin treatment and the inactivation of both proteins directly stimulates cisplatin-induced apoptosis [72]. This further suggests that these PDIs have a pro-survival role through the regulation of the cell death pathway.

Pin-pointing the exact mechanism by which PDIs contribute to tumorigenesis is impossible because PDIs interact with a wide range of proteins due to their versatile functions and have been observed to contribute to cancer progression via different pathways. The most commonly studied PDI-related signalling pathway is the Ras/Raf/MEK/ERK pathway, which is one of the key signalling pathways involved in the regulation of cell proliferation, survival and differentiation. TXNDC5 is an endo-PDI that can activate the Ras/Raf/MEK/ERK pathway and is required for TNFα-induced angiogenesis [73]. Recently, a study revealed that TXNDC5 high expression was maintained by the SREK1 variant through the interaction of nonsense-mediated decay components in hepatocellular carcinoma [74]. In addition, overexpression of ERp29 may regulate ERK signalling in mesenchymal-like breast cancer cells, which in turn increases E-cadherin and further promotes cellular transition into epithelial properties [75]. PDIA6 was reported to be involved in the Wnt/β-catenin signalling pathway. Overexpression of PDIA6 has been demonstrated to promote the proliferation and cell cycle progression of HeLa cells through activation of Wnt/β-catenin signalling by inhibiting the phosphorylation of β-catenin leading and its proteasomal degradation [76]. Studies using *Caenorhabditis elegans* have linked PDI-associated genes to the Wnt signalling pathway. The PDI-1 ortholog is crucial in specifying neuronal development and that it controls Wnt3a secretion. Mutation of the cysteine in the TRX motif to serine impairs Wnt3a secretion, suggesting that the oxidation and rearrangement of disulfide bonds mediated by PDI are essential during Wnt biogenesis in *C. elegans* [77].

Though normally residing in the ER, PDIs may translocate to the cell surface and catalyze thiol-disulfide exchange in extracellular proteins to assist in the isomerization of disulfide bonds of their interacting proteins such as metalloproteases, selectins and integrins [78]. A previous study has shown that the activation of ADAM17, a disintegrin and metalloprotease which is important for signaling at the cancer cell surface, is modulated by cell surface thiol-isomerases activity of PDIA1 [79]. In addition, PDIA1 can interact with cell surface thyroid hormone receptors via its reductase/isomerase activity suggesting its possible role in thyroid cancers [80]. PDIA1 can also be secreted extracellularly and is capable of mediating cell adhesion and migration, aiding in cancer progression and metastasis by catalyzing thiol-disulfide exchange, which further activates membrane
proteins (e.g., integrins) or proteolytic enzymes (e.g., MMPs) [81]. Another cell surface localized PDI member is PDIA6 that is highly expressed at the cell surface of human platelets in response to platelet agonists and can physically interact with the integrin B3 subunit during platelet stimulation [82]. Moreover, PDIA4 protein has been found to be secreted in the serum of Chinese adults with metabolic syndrome which elicits its potential extra-cellular role in cancer [83]. These findings set new paradigms in that PDI can have gain-of-function activities outside the ER that contribute to the hallmarks of cancer.

Among human PDIs, AGR proteins (TXNDC12, AGR2, and AGR3) are notably associated with oncogenesis. To date, the AGR gene family is the smallest PDI member which has less than 200 amino acids (Fig. 3). Since there are growing interest and studies on AGR proteins (mostly AGR2 and AGR3) in cancer; and their role in the ER and outside the ER, we ought to emphasize our review of this PDI subfamily.

AGR2

AGR2 is perhaps the most studied PDI family member and is closely related to tumorigenesis. The human homologue named AGR2 was first identified in the EsR-positive breast cancer cell lines [84]. Subsequent OMICS screening identified AGR2 as being overexpressed in the human cancers of the prostate, lung, stomach, ovarian, pancreas, esophagus, as well as head and neck [85]. It has been suggested that AGR2 plays dual roles in cancer development. The first role of ER-based AGR2 is to buffer ER proteostasis in high metabolic and proliferating cancer cells as well as to control protein secretion as widely reviewed previously [85–87]. Elevated AGR2 expression across several cancers has been shown to support the growth of cancer cells through multiple pathways such as Wnt/β-catenin and Hippo signaling pathway [88, 89]. Although AGR2 contains only a single cysteine in its TRX motif, it has been shown that it is capable of forming mixed-disulfide bonds with its substrates [90–92]. AGR2 has also been found outside the ER which was found at the cell surface and secreted into extracellular space. It has been elucidated that extracellularly localized AGR2 contributed to several hallmarks of cancer such as ECM remodeling, inflammation, metastasis, cell proliferation and angiogenesis [93–95]. For example, a recent study demonstrated that the equilibrium of AGR2 monomer–dimer influenced AGR2 secretion and that the secretion yielded pro-inflammatory phenotypes [95]. It has also been found that PDI proteins including AGR2 refluxed to the cytosol in cancer and gain new functions through a phenomenon called ER-to-cytosol Signaling (ERCYS) [96]. The reflux of AGR2 from ER to cytosol results in non-genetic inactivation of the tumor suppressor p53. There were also multiple interactomics studies aimed at expanding the search for AGR2 interaction partners to further understand the role of AGR2 in cancer. From these studies, AGR2 seems to interact largely with secreted and cell-surface proteins such as EpCAM, MUC5AC, EGFR and PROD1, suggesting that AGR2 is predominantly involved in their maturation and that the binding is crucial for their signaling [97–100].

TXNDC12 (AGR1)

TXNDC12, also known as AGR1 was initially identified from the ER lumen of healthy mice using a proteomics screen and was initially named ERp19 [101] (Table 1). TXNDC12 is the only protein within the AGR subfamily to contain dual cysteines, CGHC in the catalytic TRX motif. Interestingly, a phylogenetic tree of AGR genes across different classes of vertebrates showed that TXNDC12 is present exclusively in lower vertebrates, but lost in higher evolutionary trees [102]. TXNDC12 is believed to be the founding gene of the AGR family. Structural and biophysical studies have revealed that TXNDC12 has oxidase and isomerase activity [103–105]. TXNDC12 has been reported to form mixed disulfide bonds with substrates in the ER and in this case, TXNDC12 shows specificity to pentraxin-related protein, PTX3 during its assembly into a decamer [106]. Ectopic expression of TXNDC12 can be detected in both cell lysates and culture medium from cell lines, suggesting that this protein can be secreted extracellularly; however, the extracellular function of TXNDC12 has not been documented. TXNDC12 has also not been identified in OMICS screens of human cancer until recently. Immunohistochemical analysis and qRT-PCR of gastric cancer tissues and cell lines showed that TXNDC12 is upregulated in tumors compared to normal tissues [107]. Patients with heavy staining of TXNDC12 demonstrate poor prognoses and a positive correlation with both tumor size and lymph node metastases. TXNDC12 can also promote proliferation, migration, and invasion of gastric cancer cells while TXNDC12 knockdown significantly abolishes these effects. Interestingly over-expression of TXNDC12 significantly enhanced FAK phosphorylation at Tyr-397 and paxillin phosphorylation at Tyr118, suggesting the role of TXNDC12 in the FAK/paxillin pathway in gastric cancer [107]. This study was the first study to show that TXNDC12, like other AGR homologues, is involved in tumorigenesis at least in the gastric cancer population while its role in other types of cancer warrants further investigation.

AGR3

AGR3 is the smallest member of the PDI family containing only 166 amino acids. It was discovered as a new
protein whose sequence is highly homologous to AGR2 in a proteomics analysis from enriched plasma membrane proteins of breast cancer cell lines and was initially named BCMP11 [108]. Fluorescently labelled AGR3 demonstrated that the protein localized in secretory or endosome-like organelles in the breast cancer cell line [108]. This is another evidence of PDI member that can be captured in the plasma membrane of cancerous cell; the localization resembles a secretory molecule thus suggesting that it may function as a ligand for plasma membrane-associated proteins or maybe secreted for extracellular function. Furthermore, the same research group showed that AGR2 and AGR3 were both co-expressed in estrogen receptor (ER)-positive breast cancer and can physically interact with LY6/PLAUR Domain Containing 3 (LYPD3 /C4.4a) and extracellular alpha-dystroglycan (DAG-1) [109]. It is also interesting to note that both the AGR2 and AGR3 genes are located side-by-side in chromosome 7p21 and are in close proximity to ER binding sites, therefore it is not surprising that the genes are co-expressed particularly in ER positive breast cancer [110]. AGR3 was found to be expressed cytosolically in four human ovarian cancer subtypes; serous papillary, endometrioid, clear cell, and mucinous where the latter showed the highest AGR3 expression [111]. The expression of AGR3 was found to be associated with ER-negative ovarian cancer. In different studies, the AGR3 protein was found to be overexpressed in serous ovarian cancer and high expression of AGR3 in the serous subtype is correlated with poor prognosis [50, 112]. This result contradicts the previous study which reported that AGR3 expression is confined to the mucinous type [111]. AGR3 can also be secreted outside the cell as it was found to be secreted into blood serum and can be used as an independent prognostic factor for early screening of breast cancer patients [113]. AGR2 and AGR3 expression are coupled in ER positive breast cancer and the combination of AGR2 and AGR3 as biomarkers resulted in increased sensitivity and specificity thus suggesting that they can be used as a prognostic factor [113]. The crystal structure of AGR3 (PDBe:3PH9) has revealed that it can form an asymmetric unit consisting of two AGR3 molecules, however, there was no evidence that AGR3 can form a spontaneous dimer in solution [114].

**PDI regulation during ER stress**

The ER governs a unique environment to establish a balanced ER proteostasis. This ER proteostasis can be perturbed by physiological as well as multiple environmental and cellular signals such as high protein demand, viral infections, environmental toxins, inflammatory cytokines, and mutant protein expression resulting in an accumulation of misfolded and unfolded proteins in the ER lumen, a condition termed as ER stress [115]. It is known that under ER stress, cells activate a series of complementary adaptive mechanisms to cope with increased demands of protein folding in the ER. This adaptive system is called the unfolded protein response (UPR) which is a highly conserved signal transduction pathway to reduce unfolded/misfolded protein load and restore proteostasis [116]. When unfolded proteins accumulate in the ER, ER-resident proteins including PDIs become occupied, releasing transmembrane ER protein sensors involved in inducing the UPR. There are three key mammalian UPR sensors that comprise the endoribonuclease inositol-requiring enzyme 1-alpha (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6α) [115]. During physiological stress, upon sensing an imbalance in compartmental proteostasis, these sensors will transmit stress signals from the ER lumen to downstream signalling. This helps to reduce newly-synthesized proteins, thus facilitating the efficient degradation of superfluous and misfolded proteins in the ER [117].

An important link between ER and tumour development has been established recently [118–121]. In transformed cells like cancer, the SP is exposed to a strong environmental pressure such as hypoxia, oxidative stress or chemotherapies, aneuploidy, and high proliferation rates which together contribute to tumorigenesis. There also seems to be crosstalk between UPR with ER-associated degradation (ERAD) and autophagy that work together to remove unfolded/misfolded proteins or protein aggregates from the ER [116, 122]. The regulation by UPR is usually short-term, and prolonged activation of UPR might otherwise induce apoptosis. The prolonged activation of UPR that leads to ER stress-induced apoptosis is activated via three primary pathways, including the IRE1/ASK1/INK pathway, caspase-12 kinase pathway, and the C/EBP homologous protein (CHOP)/GADD153 pathway [123, 124].

The upregulation of PDIs is generally accompanied by the overexpression of UPR-related proteins such as BiP, GRP, IRE1α, ATF6α and eIF2a [125, 126]. For example, AGR2 expression can be controlled by the UPR upon ER stress and most likely depends on both IRE1α and ATF6α signalling [127]. Knockdowns of IRE1α and ATF6α resulted in decreased basal AGR2 mRNA expression and also prevented AGR2 induction upon chemical ER stressor treatment. Under ER stress, PDIA5 is crucial for the formation of S–S bonds in ATF6α leading to its export from the ER and activation of its target genes [128]. PDIA6 has been shown to directly interact with IRE1α and PERK and inactivates them, resulting in the limitation of UPR signalling within the physiological range [129–131].
Targeting PDIs for cancer therapeutics
It is evident that the high expression of some members of the PDI family supports cancer growth and thus gives them diagnostic/prognostic utility. This also suggests that targeting them is relevant to abrogate cancer. Interestingly, some PDIs can be secreted into the bodily fluids of cancer patients such as serum, plasma, and urine which fits a criterion to be a strong biomarker candidate. Clinical detection of PDI levels in serum could reflect PDI expression in cancer patients, thus pointing to a potential diagnostic/prognostic significance. PDIA1 has been used as a serum marker for the diagnosis of colorectal cancer [132]. Moreover, in patients with melanoma or refractory acute myeloid leukemia, PDIA1 is an immunogenic molecule that is targeted by antibodies during immune-mediated tumor breakdown [133, 134]. The same study also showed that PDIA6 can also elicit a similar antibody response, suggesting the therapeutic potential of PDIA1 and PDIA6 in hematologic malignancies. Moreover, a recent study showed that PDI inhibition resulted in the suppression of tumor cell growth and improve T cell tumor control [135].

It is worth noting that some PDIs are expressed at high levels even in normal cells (e.g., PDIA1) to maintain housekeeping activities such as substrate folding and to react to UPR signaling. Therefore, targeting these PDIs may need pharmacokinetics fine-tuning to prevent adverse systemic effects. Alternatively, a drug delivery system could be developed to specifically target PDI in cancer cells. Another important aspect to consider is that the expression landscape of PDI is different in cancer, thus targeting PDI may only work in specific types of cancer. This has been shown in the case of PDI inhibitor bacitracin which works well in inducing apoptosis in skin, breast and brain cancer cells but not in cervical cancer cells [67, 69]. For clinical applications, the most common treatment for cancer is chemotherapy. Unfortunately, chemotherapy resistance is one of the challenges in clinical cancer treatment, as several studies have shown that PDI plays a role in mediating chemoresistance in several types of cancers. For instance, it is reported that PDIA1 protein is overexpressed in ovarian cancer HeLa cells in response to aplidin, contributing to resistance to this drug. The drug resistance in the cells was abrogated through PDIA1 inhibitor bacitracin thus, implicating PDIA1 as a contributing factor to aplidin resistance. Fortunately, these data imply that combining PDI inhibitors with traditional anti-cancer compounds could resolve the chemoresistance issue and might even attain synergistic effects [136].

We summarized several other PDI agents that have been experimented on previously, their mode of action and their effectiveness in treating cancer cells in Table 2. These include antibiotics [137], sulphydryl blockers [138, 139], arsenical [140], natural and synthetic estrogentic compounds [141]. Additionally, we searched the Drugbank database (https://go.drugbank.com/) for potential drugs that could potentially be effective or repurposed to target all 21 members of the PDI family (Table 3). Only three PDI members namely, PDIA1, CASQ1, and PDIA3 could be targeted by drugs in this repository. Ribostamycin is a WHO-approved broad-spectrum antibiotic for the human application that has been repurposed to potentially inhibit PDIA1. The antibiotic was shown to inhibit PDIA1 chaperone activity but not its isomerase activity; however, the pharmacological action of the drug has yet to be elucidated [142].

As discussed previously, AGR2 is the most studied PDI member in the cancer setting and therefore, researchers have a keen interest in targeting this molecule for cancer treatment. For example, the AGR2 mouse monoclonal antibody has already been developed and was shown to suppress the growth of breast cancer cells [180]. A further study developed a humanized version of the same antibody and demonstrated that the antibody was able to inhibit tumor growth in an ovarian cancer xenograft model [181]. Another study had developed the same monoclonal antibody was also able to inhibit lung cancer growth, suppressed tumor metastasis and prolong survival in mice [182]. Additionally, monoclonal antibodies in the form of the synthetic single-chain variable fragment (scFv) isolated from antibody phage display are currently being developed and current data demonstrated that the scFv targeting AGR2 was able to bind the N-terminal of AGR2 with high affinity [183].

Concluding remarks
The founding member PDIA1 was discovered almost 60 years ago [184], and the idea of the PDI family was introduced in 1994 with only 4 members [185]. Since then, the family has been expanded to include new members with different structural features, sizes and functions but having the capability to assist in protein folding especially vis-à-vis disulfide bond formation. Owing to this capability, artificial mini proteins have been developed to resemble the PDI-like catalytic activity for promoting the folding process in preparations of peptide-based drugs [186]. Though PDI proteins are crucial in the normal physiology of the ER by ensuring proper folding and maturation of substrate proteins before they are exported to their destined locations, it has been found that several members of the PDI play roles in cancer as discussed in the review. It is not surprising that some PDIs are elevated in cancer to cater for the rapid growth of cancer cells due to higher protein synthesis demand in cancer as opposed to normal cells. PDI proteins have also been
| Name                  | IC50 (micromolar) | Selectivity       | Reversibility | PDI inhibition activity                                                                 | References               |
|-----------------------|-------------------|-------------------|---------------|----------------------------------------------------------------------------------------|--------------------------|
| Acrolein              | 10 (in pH 6.3)    | Nonspecific       | Irreversible  | N/A                                                                                     | [143]                    |
| Bacitracin            | 90                | Nonspecific       | Irreversible  | • Apoptosis increased together with other drugs in melanoma cells                       | [144–148]               |
| Bacitracin A (major analogue) | 590        |                   |               | • Glioma cell progression and invasion were reduced                                     | [149, 150]               |
| Bacitracin B          | 1050              |                   |               | • Inhibits virus entry                                                                  | [149–153]               |
| Bacitracin F          | 20                |                   |               | • Inhibits platelet accumulation                                                        | [154, 155]               |
| Bacitracin H          | 40                |                   |               | • Aggregation of Cu/Zn superoxide dismutase increases                                   | [156–161]               |
| Bepristat 2a         | 0.3–2.1           | b’ domain of PDIA1| Reversible    | • Prevent adhesion of MDA-MB-231 and MCF-7 cells to collagen, endothelium and fibronectin| [162, 163]               |
| Cysteamine           | 66                | Nonspecific       | Irreversible  | • Prevents polyQ-induced apoptosis in a PC12 cell model of Huntington’s Disease         | [164]                    |
| DNTB                  | 100               | Nonspecific       | Irreversible  | • Inhibits diphtheria toxin activation                                                 | [153, 160, 165]         |
| Name                          | IC₅₀ (micromolar) | Selectivity | Reversibility | PDI inhibition activity | References |
|-------------------------------|-------------------|-------------|---------------|-------------------------|------------|
| Estrogens: E₁, E₂, DES and E₃ | At 1 micromolar > 30% inhibition | Nonspecific | Reversible | In-vitro In-vivo | [166] |
| Iodoacetamide                 | 8 (pH 6)          | Nonspecific | Irreversible |                     | [143] |
| Juniferdin                    | 0.156–3           | PDIA3 and PDIA4 | Reversible |                      | [167] |
| NEM                           | 8                 | Nonspecific | Irreversible |                      | [143] |
| PACMA 31                      | 10                | Shows selectivity for Cys53 of α domain | Irreversible | Inhibits in ovarian cancer cell lines (OVCAR-8, NCI/ADR-RES, HEY and OVCAR-3) | Accumulates in tumor and suppresses tumor growth in a mouse xenograft model of ovarian cancer; no significant toxicity towards normal tissues | [168] |
| PAO                           | 85                | Low specificity reaction with CXXC motif | Irreversible | •Induces rapid shedding of L-selectin from isolated neutrophils •Inhibits PDI-catalyzed reductive release of acid-soluble \(^{125}\)I-tyramine-SH from surface-bound \(^{125}\)I-tyramine-SS-poly(D-lysine) (IC₅₀ = 10 micromolar) •Effective before or during HIV-1 infection •Non-effective after infection progressed in P4, PM1, H9, TGS and macrophage-depleted peripheral blood mononuclear cells | [168–172] |
| Quercetin-3-rutinoside (rutin)| 6.1               | PDIA3, PDIA4, PDIA6, thioredoxin and thio- redoxin reductase | Reversible | •Inhibits platelet aggregation and endothelial cell-mediated fibrin generation Inhibits thrombus formation | [146, 173] |
| Name                                    | IC_{50} (micromolar) | Selectivity  | Reversibility | PDI inhibition activity                           | References                  |
|-----------------------------------------|----------------------|--------------|---------------|--------------------------------------------------|-----------------------------|
| Quercetin-3-glucoside (isoquercetin)    | 9.2                  | Plasma PDI   | Reversible    | •Increased plasma PDI inhibitory activity         | [174, 175]                 |
|                                         |                      |              |               | •Decreases thrombin production                     |                             |
| RB-11-ca                                | 40                   |              | Irreversible  | •Cytotoxic in HeLa cells with EC_{50} = 23.9 micro‑molar | [176]                      |
| Ribostamycin                            | Sufficient inhibition of PDI with a molar ratio of 1001 (Kd = 3.19 × 10^{-4} M) | Nonspecific | Reversible    |                                                  |                             |
| Thiomuscimol                             | 23                   | Nonspecific  | Irreversible  | •Prevents polyQ-induced apoptosis in a PC12 cell model of Huntington’s Disease | [142]                      |
|                                          |                      |              |               |                                                  |                             |
| 16F16                                   | 63                   |              | Irreversible  | •Inhibits apoptosis in a PC12 cell model of Huntington’s Disease | [164]                      |
|                                          |                      |              |               | Prevents neurotoxicity in medium spiny neurons in the striatal region of brain slices |                             |
| E64FC26                                 | 1.9–25.9             | PDIA1, PDIA3, PDIA4, TXNDC3, PDIA6 |              | •Inhibit tumor cell growth and T cell tumor control | [135, 177]                 |
|                                          |                      |              |               | Improved survival and enhanced the activity of bortezomib in multiple myeloma |                             |
| CCF642-34                               | < 5                  | PDIA1        |              | •Induces apoptosis in myeloma cells               | [178, 179]                 |
implicated in a wide range of diseases other than cancer including neurodegenerative, cardiovascular, inflammatory bowel, infectious, metabolic and bone diseases [187–189]. These diseases are caused or associated with protein misfolding, accumulation, aggregation or assembly that requires elevated and robust folding catalysts like PDIs. Therefore, the development of drugs that target these pathways will be of benefit not only for cancer therapy but would be applicable for these other associated diseases as well.

The known and proposed roles PDI proteins in cancer setting are summarized in Fig. 4. Attention is now turning to the non-ER localized function and in particular the roles of cell surface and secreted PDIs [83, 190, 191]. This is interesting because PDI proteins are crucial for ER function and integrity, and the localization of

Table 3  Approved and investigational drugs targeting PDIs

| Target   | Drug name                                                                 |
|----------|---------------------------------------------------------------------------|
| PDIA1    | Ribostamycin, copper, zinc, artemimol, zinc acetate, zinc chloride, zinc sulfate |
| CASQ1    | Calcium citrate, calcium levulinate, calcium phosphate, calcium phosphate dihydrate |
| PDIA3    | Copper, zinc, zinc acetate, zinc chloride, zinc sulfate                     |
| CASQ2    | Calcium citrate, calcium phosphate, calcium phosphate dihydrate             |
PDI proteins outside the ER has sparked an interest in the field because it has been shown that the presence of selected PDI proteins in extracellular can contribute to the emergence of cancer. The extracellular roles of PDI need to be further examined to isolate the key players and pathways that can contribute to cancer. Another aspect that can be pursued is to closely look at the three-dimensional (3D) structure of each PDIs. The advancement of high-resolution structural biology techniques like cryo-electron microscopy and structural bioinformatics using prediction-based methods (e.g., AlphaFold [192]) could give additional insight into PDI enzymatic and chaperone activities. Moreover, cancer cells could be highly dependent on molecules or pathways that support their growth and progression. Therefore, targeting the interfaces between proteins (i.e., protein–protein interaction network) had been proposed as a cancer therapeutic option. In this case, it is worth it to (1) expand the PDIs client proteins and identify the PDIs protein–protein interaction hubs; and (2) screen and validate drugs/inhibitors that could collapse these protein–protein interaction interfaces. Also, gene targeting technologies such as the CRISPR-based genome editing tools could be employed to identify the PDIs’ downstream targets in cancer cells. Finally, significant attention should also be drawn to the other PDI family members, especially to those that are least studied. A comprehensive understanding of the biology of the PDI family members could shed light on how PDIs deregulation would promote the pathogenesis of cancers as well as other related protein-folding diseases.

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