

The Role of POPDC1 in the Progression of the Malignant Phenotype

Steven J Tucker, Alina J Zorn

University of Aberdeen

Conflicts of Interest: no conflicts

Abstract

The Popeye Domain Containing Protein 1 (POPDC1), a tight junction-associated transmembrane protein with a unique binding site for cAMP, has been shown to act as a tumour suppressor in cancer cells. Through interaction with many downstream effectors and signalling pathways, POPDC1 promotes cell adhesion and inhibits uncontrolled cell proliferation, epithelial-to-mesenchymal transition, and metastasis. However, POPDC1 expression is downregulated in many types of cancer, thereby reducing its tumour-suppressive actions.

This review discusses the role of POPDC1 in the progression of the malignant phenotype and highlights the broad range of benefits POPDC1 stabilisation may achieve therapeutically. Cancer stem cells (CSC) are a key hallmark of malignancies and commonly promote treatment resistance. This article provides a comprehensive overview of CSC signalling mechanisms, many of which have been shown to be regulated by POPDC1 in other cell types, thus suggesting an additional therapeutic benefit for POPDC1-stabilising anticancer drugs.

Keywords:

POPDC1, cAMP, cancer, cancer stem cells, proliferation, metastasis, epithelial mesenchymal transition

1. Introduction

The Popeye Domain Containing Protein 1 (POPDC1) was discovered in 1999 and research over the last 20 years has shown it to be implicated in the progression of malignancies. Initially discovered in cardiac tissue, POPDC1 (also known as blood vessel epicardial substance (BVES)) has a wide tissue distribution and is found in smooth and skeletal muscle, the brain, gastrointestinal tract and liver (Han *et al.*, 2019). It is also present in many cancers, such as breast cancer (BC), gastric cancer (GC), colorectal cancer (CRC), non-small cell lung carcinoma (NSCLC), hepatocellular carcinoma (HCC) and uveal melanoma (UM) (Han *et al.*, 2019). Whilst POPDC1 proteins regulate signalling pathways in cardiac and skeletal muscle tissue (Brand, 2018), the focus of this review will be their role as tumour suppressors. Dysregulation of POPDC1 expression in cancer cells may offer an interesting and novel therapeutic target in the treatment of cancer, and will be discussed in the following article.

1.1. The Structure of the POPDC1 Protein

Popeye domain containing proteins exist as three isoforms: POPDC1, POPDC2 and POPDC3. Structurally, these isoforms differ in their amino acid chain length: 360, 364 and 291 residues, respectively (Brand and Schindler, 2017). It has been suggested that these three subtypes and their splice variants exist to facilitate tissue-specific target interaction as a result of truncated binding regions (Benesh *et al.*, 2013). In addition to the variations in amino acid chain length, the POPDC2 and POPDC3 isoforms differ in their intracellular phosphorylation sites (Brand and Schindler, 2017) and have only one extracellular glycosylation site at aa4 (Uniprot, 2018. Available at: <https://www.uniprot.org/uniprot/Q9HBU9> and <https://www.uniprot.org/uniprot/Q9HBV1>). Interestingly, whereas the POPDC2 gene is located on 3q13.33, the POPDC1 and POPDC3 gene are found on the same chromosome (6q21), which suggests that the POPDC3 gene may have arisen as a result of a tandem-duplication of the POPDC1 gene (Schindler *et al.*, 2012).

POPDC1 has been extensively studied over the last 20 years since its discovery, while less work has been done on elucidating the role POPDC2 and POPDC3 may play in health and disease. POPDC2 and POPDC3 proteins are highly expressed in both skeletal muscle and cardiac tissue (Brand and Schindler, 2017) and various mutations in these isoforms have been identified that are associated with tissue dysfunction at these locations (Table 1) (Vissing *et al.*, 2019; Amunjela *et al.*, 2019). The data in Table 1 suggests that the action of POPDC2 seems more confined to striated muscle tissues (Brand, 2019), whilst POPDC3 is dysregulated across a range of malignant phenotypes. In line with their similar genetic origin outlined above, the role of POPDC3 resembles that of POPDC1 in cancer cells. For example, downregulation occurs in a similar manner (e.g. promoter hypermethylation) and POPDC3 has a tumour-suppressive role in gastric cancer (Kim *et al.*, 2010). However, whilst POPDC1 shows tumour-suppressive properties across most cancers, POPDC3 seems to have more restricted oncogenic roles in breast cancer, HNSCC, oesophageal and lung cancer.

A summary of the data gathered on POPDC2 and POPDC3 to date is presented in Table 1. Due to the greater body of evidence and understanding of the POPDC1 protein, this review will focus on the role of POPDC1 in the progression of the malignant phenotype.

POPDC1 is a transmembrane protein (Figure 1) and is present in all muscle types, various parts of the GI tract and most importantly for this review, many subtypes of cancer.

POPDC1 has a short extracellular N-terminal domain (42 amino acids long) with two asparagine residues that are subject to N-glycosylation (residues 2 and 30) (Uniprot, 2018, Available at: <https://www.uniprot.org/uniprot/Q8NE79>). Variation in the location of N-glycosylation sites (e.g. aa20 and aa27) (Knight *et al.* 2003; Parang *et al.*, 2018; Amunjela *et al.* 2019), may occur in different splice variants of the POPDC1 protein. The level of glycosylation seems to differ between tissue types, with the extent of glycosylation shown to be greater in skeletal muscle and brain tissue than cardiac tissue, which may be of functional relevance (Vasavada *et al.*, 2004; Brand, 2019). The extent of POPDC1 glycosylation in cancer cells and the functional relevance of this has yet to be investigated.

This extracellular sequence is followed by three transmembrane domains and a larger, intracellular carboxy-terminal domain (CTD) (aa114-360). The intracellular CTD contains the highly conserved POPEYE domain (aa172-266), which contains the binding sites for cAMP and other downstream effectors, such as TREK-1, a potassium ion channel, caveolin 3 (CAV-3), a plasma

membrane protein, the guanine exchange factors GEFT and GEFH, the PR61 α subunit of protein phosphatase 2, and ZO-1, a tight junction-associated protein (Parang *et al.*, 2018) (Table 2).

The cAMP binding domain, also referred to as the phosphate binding cassette (PBC) consists of two highly conserved tetrapeptide sequences (DSPE and FQVT) that are linked by a sequence of variable length (Brand, 2018). The three-dimensional structure of the PBC is very similar to that of other cAMP-activated proteins such as PKA, however, there is a large difference in the amino acid sequence encoding this domain (Schindler *et al.*, 2012), which may offer an avenue for therapeutic exploitation.

POPDC1 forms a homodimer in the cell membrane mediated by a disulfide bond established between intracellular cysteine residues. This is facilitated by two highly conserved lysine (K) residues at positions 272 and 273 (Kawaguchi *et al.*, 2008; Russ *et al.*, 2011). Dimerisation seems to be essential for protein trafficking to the membrane as well as protein function whilst in the membrane leaflet. This is evidenced by loss or mutations of KK residues leading to reduced membrane localisation and loss of cell adhesion (Kawaguchi *et al.*, 2008 and Brand, 2018). However, some evidence has shown that POPDC1 can still form a homodimer in the absence of the KK motif (Schindler and Brand, 2016).

In vitro studies by Osler *et al.* (2005) using epicardial mesothelial cells showed that before cell-cell contact was established, POPDC1 was localised in the cytoplasm. Once contact between neighbouring cells formed, POPDC1 inserted itself into the cell membrane and co-localised with other junctional proteins, resulting in cell adhesion (Osler *et al.*, 2005; Parang *et al.*, 2018).

Furthermore, POPDC1 proteins that are no longer inserted into the cell membrane (e.g. in BC or HCC cells) have been shown to accumulate intracellularly (Russ *et al.*, 2011; Han *et al.*, 2015; Amunjela *et al.*, 2019), but it remains unclear whether this accumulation is a pathway to POPDC1 degradation or a progressive storage mechanism. The potential effect of nuclear POPDC1 on gene transcription has also yet to be elucidated (Amunjela *et al.*, 2019).

1.2. Hallmarks of Cancer

Cancer, the inappropriate and uncontrolled growth of cells which can lead to the formation of malignant tumours, is one of the leading causes of death worldwide.

Over 20 years ago, Hanahan and Weinberg defined six key hallmarks which characterise the malignant phenotype of this disease. These include self-sufficient growth signalling (cancer cells don't require endogenous signals, but generate their own growth factors to stimulate proliferation), acquired resistance to growth-inhibiting signals, evasion of apoptosis, replicative immortality (by reactivating telomerase reverse transcriptase (TERT) to prevent telomere shortening), sustained angiogenesis (to maintain oxygen and nutrient supply allowing tumour growth) and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

A decade later, the authors extended their work by four enabling characteristics of cancer, which are not unique to cancer cells, but help drive disease progression. Genome instability and an inflammatory tumour environment have been shown to promote malignant growth, whilst altered cellular energetics and immune system evasion (due to surface antigen loss and cancer stem cell formation), further enable cancer progression (Hanahan and Weinberg, 2011). The most relevant hallmarks in the context of this review are uncontrolled cell proliferation, loss of cell adhesion and

metastasis, as well as the presence of cancer stem cells (CSC), which drive malignancy, treatment resistance and cancer recurrence (Du *et al.*, 2019).

The treatment options for cancer are based on inhibiting these processes, however, current therapies show varying degrees of success. As the POPDC1 protein has been shown to play a role in cancer cell proliferation, loss of adhesion and metastasis (Figure 2), this protein presents an exciting target for developing novel anti-cancer drugs.

1.3. Signalling Mechanisms and Downstream Targets of POPDC1

The POPDC protein family is one of the five downstream targets of cyclic adenosine monophosphate (cAMP), the other four being PKA (protein kinase A), Epac (exchange protein directly activated by cAMP), HCN (hyperpolarisation-activated cyclic nucleotide-gated channel) and CRIS (cyclic nucleotide receptor involved in sperm function) (Schindler and Brand, 2016). cAMP binds to the PBC of the POPEYE domain (discussed above) and leads to increased protein expression, stabilisation, and activation (Amunjela *et al.*, 2019).

Studies have shown that increased cellular levels of cAMP correlate with increased apoptosis and reduced proliferation, invasion, and metastasis in cancer cells. As other downstream effectors of cAMP such as PKA and Epac have been shown to promote these hallmarks, this suggests that downstream pro-apoptotic effects of increased cAMP in cancer cells could, at least in part, be mediated by the actions of POPDC1 proteins (Amunjela *et al.*, 2019).

POPDC1 has been shown to influence signalling pathways, such as the Wnt-pathway, transcription factors (e.g. c-Myc), and to interact with proteins including caveolin-3 (CAV-3), TREK1 (two-pore domain potassium channel), tight junction-associated proteins (e.g. ZO-1, occludin), guanine nucleotide exchange factors (GEFT, GEFH) (Smith *et al.*, 2008; Parang *et al.*, 2018) and the vesicular transport protein VAMP3 (Han *et al.*, 2019) (Figure 2; Table 2).

Whilst the interaction of POPDC1 with TREK1, CAV-3 and Bnip3 has thus far mainly been demonstrated in cardiac and skeletal muscle function (Brand, 2019), recent studies have found that TREK1 is overexpressed in prostate cancer cells, CAV-3 is upregulated in anaplastic thyroid carcinoma (Han *et al.*, 2019) and Bnip3 levels are increased in breast cancer and NSCLC (Lee *et al.*, 2014). However, the presence of POPDC1 and interaction with these proteins in cancer cells has yet to be established. The known effects of POPDC1 on downstream targets in cancer cells will be discussed in this review.

1.4. Downregulation of POPDC1 in Cancer Cells

The downregulation of POPDC1 proteins has been demonstrated in a large range of cancer cell types. Loss of POPDC1 expression has been proposed to be initiated by four main mechanisms. Firstly, hypermethylation of the cytosine-phosphate-guanine islands of the POPDC1 gene promoter has been demonstrated in a myriad of cancers (Table 3) and has hence been suggested as a biomarker for early detection of cancer (Parang *et al.*, 2018). Secondly, the under-expression of miRNA-122 (Wang *et al.*, 2014) and thirdly, over-expression of netrin-1 (Han *et al.*, 2015) has been shown to suppress POPDC1 expression. Finally, increased EGFR stimulation leads to a significant suppression of POPDC1 activity, most likely as a result of phosphorylation of the POPDC1 C-terminal domain (Amunjela and Tucker, 2017a). The mechanisms implicated in different cancers are shown in Table 3.

The loss of POPDC1 expression and its membrane integration means it is no longer able to influence cell adhesion and interact with the various signalling pathways and proteins outlined above (Table 2). This article will discuss the effects this has on cancer cells proliferation, transformation and migration.

2. The Role of POPDC1 in Cancer Cell Proliferation

Uncontrolled cell proliferation is a key hallmark of cancer progression (Hanahan and Weinberg, 2000) and is often a result of dysregulated growth factor signalling. Not only do cancer cells increase their sensitivity to exogenous growth-stimulating factors, but they also acquire the ability to generate their own growth signals that act in an autocrine manner to promote proliferation. Moreover, signalling pathways in cancer cells often become dysregulated, resulting in reduced transcription of growth-inhibiting genes, and increased transcription of growth-promoting genes (Hanahan and Weinberg, 2011).

Popeye domain containing protein 1 has been implicated in the regulation of numerous signalling pathways involved in cell proliferation. Previous studies have shown that, at least in breast cancer cells, increased cAMP levels lead to higher POPDC1 expression and protein stabilisation, resulting in reduced cell proliferation and migration (Amunjela and Tucker, 2017b). The mechanisms of POPDC1-mediated control of cancer cell proliferation will be discussed in the following section.

2.1. Interaction with ZO-1 and ZONAB

POPDC1 has been shown to interact with ZO-1 (aka tight junction protein 1, TJP1) to maintain cell adhesion, tight junction-associated signalling and prevent migration (Russ *et al.*, 2011). This interaction allows ZO-1 to recruit ZONAB/DbpA to the tight junction and prevent its nuclear localisation (Amunjela *et al.*, 2019).

If POPDC1 protein expression is lost in cancer cells, ZONAB/DbpA can translocate to the nucleus. ZONAB/DbpA is a γ -box transcription factor and, via interaction with cell division kinase 4 (CDK4), it increases transcription of HER2, cyclin D1 and PCNA (Jayagopal *et al.*, 2011; Amunjela *et al.*, 2019), allowing entry into the cell-cycle and progressive cell proliferation.

When looking at the interaction of POPDC1 with these proteins in cancer cells, ZO-1 and ZONAB interaction has been demonstrated in uveal melanoma (Jayagopal *et al.*, 2011), and a correlation between POPDC1 and ZO-1 loss has been observed in HCC (Han *et al.*, 2019). The POPDC1/ZO-1 interaction in other cancer types requires future investigation.

2.2. Interaction with GEFT and GEFH

Rac/Cdc42 specific exchange factor, a guanine nucleotide exchange factor more commonly referred to as GEFT can interact (via aa300-aa450) with POPDC1 via its C-terminal residues aa250-aa300 (Smith *et al.*, 2008). GEFT preferentially binds to and increases Rac1 and Cdc42 (cell division control protein 42) activity, which promote cell proliferation. In addition, activation of Rac1 and Cdc42 disrupts cell adhesion and promotes cell migration, which is discussed later in this review.

POPDC1 has been shown to negatively regulate GEFT (Smith *et al.*, 2008) and thereby reduce Rac1 and Cdc42 activity, supporting its role as a tumour suppressor (Amunjela *et al.*, 2019). High GEFT activity has been demonstrated in brain tumours (Guo *et al.*, 2003), but the GEFT/POPDC1 interaction has so far only been demonstrated in fibroblasts (NIH T3T cells) (Smith *et al.*, 2008) and human corneal epithelia, where POPDC1 interacting with two different GEFs, GEFT and GEFH, seems to promote tight junction (TJ) formation by reducing RhoA activity (Russ *et al.*, 2010; Russ *et al.*, 2011).

When epithelial cell confluence has been established through formation of desmosomal, tight and adherens junctions, the activation of RhoA signalling that stimulates further cell proliferation is inhibited by GEFH and cingulin (TJ-associated protein) (Parang *et al.*, 2018). Hence, loss of TJ formation in cancer cells that under-express POPDC1 could re-establish RhoA signalling and induce cancer cell proliferation.

2.3. c-Myc Pathway

c-Myc is a pro-oncogenic transcription factor commonly overexpressed in cancer cells. Active c-Myc plays an important role in the transcription of proliferative genes, cell-cycle control, cell differentiation, and EMT (Parang *et al.*, 2017; Swan *et al.*, 2019).

Phosphorylation of the S62 residue of c-Myc by MAPK keeps it in an inactive state and thereby maintains a steady c-Myc concentration in the cell. Further phosphorylation of the T58 residue by GSK3 β allows subsequent dephosphorylation of the S62 residue by the PR61 α subunit of serine-threonine protein phosphatase 2A (PP2A). PP2A is a heterotrimeric protein made up of a regulatory, structural, and catalytic subunit (Parang *et al.*, 2017). Once the S62 residue has been dephosphorylated, c-Myc is ubiquitinated and targeted for proteasomal degradation.

POPDC1 has been shown to increase the negative regulation of c-Myc. It interacts with both c-Myc and the PR61 α (aka PPP2R5A) subunit of PP2A via a 15 amino acid sequence on its C-terminal end (aa330-aa345), directing it towards the dual-phosphorylated S62/T58 c-Myc and promoting S62 dephosphorylation (Parang *et al.*, 2017). This increases degradation of pro-mitotic c-Myc.

Loss of POPDC1 in cancer has been shown to decrease T58 phosphorylation, reduce c-Myc ubiquitination and increase active c-Myc (Parang *et al.*, 2017). Together, this leads to increased proliferative signalling.

2.4. Wnt/ β -catenin Pathway

The Wnt/ β -catenin pathway is a major cell signalling pathway where mutations in pathway components have been linked to the progression of several cancer types, including gastrointestinal cancers, melanoma, leukaemia and breast cancer (Zhan *et al.*, 2017).

The Wnt signalling pathway can be activated in a β -catenin-dependant (canonical pathway) or independent (non-canonical pathway) manner. Usually, in the absence of a Wnt-ligand, β -catenin is phosphorylated and maintained in an inactive state by protein kinases GSK3 β and CK1 α , both part of the axin2 complex (also referred to as the destruction complex). Another enzyme in this complex, β -TrCP, ubiquitinates β -catenin targeting it for proteasomal degradation (Zhan *et al.*, 2017).

Wnt ligands (e.g. Wnt3a or Wnt1), bind and activate the frizzled (Fzd) protein, and the neighbouring co-receptor lipoprotein receptor-related protein 6 (LRP6). The LRP6 receptor is in turn phosphorylated by GSK3 β and CK1 α , recruiting dishevelled (Dvl) proteins to the membrane where they polymerise. This leads to the inactivation of the axin2 complex, and β -catenin can no longer be inactivated (Zhan *et al.*, 2017). β -catenin can then translocate to the nucleus, where it binds to lymphoid and T-cell enhancing factors (LEF/TEF) and other transcription factors (e.g. cyclin D1), leading to the transcription of proliferative genes. It also triggers transcription of genes involved in metastasis, which will be discussed later. The activation of the non-canonical pathway and its involvement in cancer cell metastasis will also be examined within this review.

POPDC1 has been shown to play an important role in regulating the Wnt pathway (Thompson *et al.*, 2019) by interacting with the co-receptor of the canonical pathway: LRP6. POPDC1 binding to the LRP6 receptor, which occurs through the intracellular/transmembrane part of LRP6, keeps β -catenin in its inactivate state preventing activation of its downstream targets. The loss of POPDC1 protein expression in cancer cells (see 1.4.) has been shown to enhance Wnt signalling (Parang *et al.*, 2017). Studies have shown that loss of POPDC1 leads to LRP6 phosphorylation (Ser1490) and recruitment of axin2 to the cell membrane, stabilising β -catenin. This results in increased β -catenin signalling and leads to cellular changes that promote proliferation and metastasis (Thompson *et al.*, 2019). Hence, stabilising the POPDC1 protein to maintain LRP6 interaction may be a good method to reduce cancer cell proliferation.

The Wnt signalling pathway is further regulated by protein phosphatase 2A (PP2A) (Thompson and Williams, 2018). Whilst this regulation seems to occur in both a positive and negative manner, only the negative regulation of Wnt signalling has been shown to be mediated through the PR61 α subunit. This subunit promotes β -catenin degradation through an APC-dependant signalling complex (Thompson and Williams, 2018). As previously discussed in 2.3., POPDC1 interacts with this specific subunit of the PP2A protein and promotes c-Myc degradation. It would be interesting to further investigate and confirm if POPDC1/PR61 α interaction has a role in also negatively regulating Wnt signalling.

2.5. Bnip3

Bnip3 (BCL2 and adenovirus E1B 19-kDa-interacting protein 3) is a mitochondrial, membrane-bound protein that is part of the Bcl2 family. The Bcl2 protein family balances cell proliferation and apoptosis; a balance often dysregulated in cancer cells (Hanahan and Weinberg, 2011). Once activated, Bnip3 mediates opening of mitochondrial permeability transition pores and interacts with BAX/BAK proteins to induce apoptosis. Marked increases in Bnip3 expression in hypoxic areas of solid tumours has been shown in BC and NSCLC, but has been shown to be suppressed in other cancer types (especially pancreatic cancer, CRC and GC) to prevent apoptosis (Lee *et al.*, 2006)

POPDC1 has been shown to suppress Bnip3 activity in cardiac myocytes, and loss of POPDC1 resulted in Bnip3 upregulation and increased apoptosis (Kliminski *et al.*, 2016). It was further shown that reduced POPDC1 expression also reduced expression of Bnip3. However, the role of the POPDC1/Bnip3 interaction in cancer cells remains to be investigated.

2.6. Outlook - Inhibiting Cancer Cell Proliferation by Stabilising POPDC1

From the data explored above, it is evident that increasing expression or stabilising existing POPDC1 protein in cancer cells could prevent aberrant proliferation through a number of mechanisms.

Firstly, cell proliferation could be inhibited by stabilising POPDC1-mediated cell adhesion. This could maintain POPDC1 interaction with tight junction (TJ) proteins such as ZO-1 and occludin and prevent activation of ZONAB and RhoA and subsequent transcription of proliferative genes. TJ loss has been established in a myriad of cancers (Salvador *et al.*, 2016), and leads to the activation of many downstream signalling pathways (Bhat *et al.*, 2018). Although this makes the TJ an interesting target in cancer treatment (Shah, 2012), a current lack of understanding of how different TJ components are involved in regulating these pathways in cancer poses a barrier to investigating TJ proteins specifically as therapeutic targets (Bhat *et al.*, 2018).

Mechanisms by which the GEFT pathway could be targeted for anticancer drug development is not only apparent in cell proliferation but is also strongly implicated in cancer metastasis. Novel approaches to targeting this pathway and potential roles for POPDC1 stabilisation are discussed in the respective sections below.

c-Myc Signalling Pathway

The c-Myc pathway has long been implicated as a promising anti-cancer drug target. This relates to the Myc oncogene being implicated in driving many hallmarks of cancer (proliferation, suppressed apoptosis, altered cell metabolism) and its dysregulation in over 50% of all cancers (Chen *et al.*, 2018). However, targeting c-Myc specifically has proven difficult due to its nuclear localisation as a transcription factor and its “undruggable” protein structure. Hence, alternative parts of the c-Myc pathway must be investigated for drug development (Chen *et al.*, 2018).

As described above, PP2A is crucial for c-Myc ubiquitination and subsequent degradation, and various approaches to increase PP2A activity are currently being investigated for therapeutic use (Remmerie and Janssens, 2019). It has been shown that POPDC1 interacts with the PR61 α subunit of PP2A to increase c-Myc inactivation. Therefore, we hypothesise, that stabilising POPDC1/PP2A interactions in cancer cells may be a novel therapeutic approach for promoting c-Myc degradation and reducing its impact in malignant progression.

Wnt signalling Pathway

Wnt pathway involvement in cancer cell proliferation has also been largely investigated in the development of new anticancer drugs as Wnt signalling is important for maintaining global tissue homeostasis. However, pharmacologically targeting this pathway may bring various unwanted side-effects due to the ubiquitous nature of the signalling pathway (Raisch *et al.*, 2019). Hence, the LRP6 co-receptor (described in detail above), which is frequently overexpressed in CRC, hepatic cancer, BC and pancreatic ductal adenocarcinoma and is associated with increased β -catenin signalling, poor prognosis and chemoresistance, has been explored as a novel, more specific therapeutic target to inhibit the Wnt pathway (Raisch *et al.*, 2019). Thus far, only the extracellular binding sites of LRP6 have been targeted, either by inhibiting Wnt secretion to reduce receptor activation (using porcupine inhibitors), by increasing Dickkopf-related protein 3 concentration (a LRP5/6 antagonists), or by using monoclonal antibodies that prevent Wnt binding to the extracellular binding sites of LRP6 (Raisch *et al.*, 2019). These approaches each come with limitations relating to ligand and/or receptor specificity. Therefore, decreasing LRP6 activation

intracellularly through stabilisation of POPDC1 and allowing interaction with the intracellular portion of LRP6 to prevent β -catenin activation, could be a novel, specific approach in drug development to dampen down Wnt signalling and reduce cancer cell proliferation.

3. Role of POPDC1 in Cell Adhesion and Metastasis

Another important hallmark of cancer is the loss of cell adhesion, which allows cells to gain motility and spread to distant sites in the body (metastasise). Cancer cell metastasis presents one of the greatest hurdles in successful anti-cancer treatment, especially as secondary tumours preferentially arise in tissues that are very difficult to target with traditional therapy (e.g. brain and bone). It is estimated that metastasis is the cause of around 90% of cancer fatalities (Guan, 2015). This highlights why early intervention and prevention of metastasis is key to therapeutic success.

Metastasis occurs in four main stages: loss of cell adhesion; cytoskeletal remodelling and gain of cell motility through EMT; intravasation and entry into the vascular or lymphatic system; and finally, extravasation (re-entry into surrounding tissue at a distal site).

POPDC1 proteins have been shown to play an important role in cell adhesion and cancer cell metastasis. The following will review the various signalling mechanisms and downstream targets of POPDC1 implicated in these processes.

3.1. Cell Adhesion

As previously discussed, POPDC1 has been shown to play an important role in maintaining epithelial tight junctions and cellular adhesion by interacting with occludin, ZO-1 and the Wnt signalling pathway. The latter two prevent activation of ZONAB and β -catenin-induced gene transcription, respectively, which lead to cytoskeletal rearrangement, disruption of tight junctions and increased cell motility (Parang *et al.*, 2018).

A loss of cell adhesion and tight junction formation in the absence of POPDC1 expression has been demonstrated in experiments using POPDC1 knock-outs (Parang *et al.*, 2018) and mutated ($K^{272/273}$ mutation preventing dimerization) POPDC1 proteins (Kawaguchi *et al.*, 2008). These highlight that the reduced expression of POPDC1 associated with cancer cells may be an important step in mediating loss of cell adhesion, the first stage in the metastatic cascade.

3.2. Epithelial-to-Mesenchymal Transition (EMT)

A further key step in the metastatic cascade, the gain of cell motility, is a result of epithelial-to-mesenchymal transition (EMT) which has been shown to be key to progression in all cancer types (Dongre and Weinberg, 2019). Three types of EMT exist: Type 1 and 2, respectively, are important for embryonic development and wound healing, while Type 3 EMT has been shown to be a central component in cancer metastasis.

The reverse process of EMT, mesenchymal-to-epithelial transition (MET) is undergone after extravasation into the tissue at a secondary site. In undergoing MET, cancer cells can re-gain adhesive properties and start proliferating to form secondary malignant tumour masses (Hanahan and Weinberg, 2011).

EMT is initiated by the expression of EMT-inducing transcription factors (EMT-TFs), e.g. SNAIL, SLUG and TWIST. These suppress the expression of proteins associated with the epithelial phenotype (e.g. E-cadherin, occludins, $\alpha 6 \beta 4$ integrins) and activate expression of mesenchymal proteins such as N-cadherin, vimentin, fibronectin, $\beta 1$ - and $\beta 3$ -integrins. Key activators of EMT-TFs in cancer cells are TGF β , β -catenin via the Wnt signalling pathway, as well as the tumour microenvironment (Dongre and Weinberg, 2019). Examples of cytokines and chemokines released from the cancer-associated fibroblasts and immune cells of the tumour microenvironment include transforming growth factor β (TGF β), interleukin-6 (IL-6), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), tumour necrosis factor (TNF), and hepatocyte growth factor (HGF) (Dongre and Weinberg, 2019).

It has been shown that some carcinoma cells don't undergo complete transition to the mesenchymal state (unlike during embryogenesis (type 1 EMT) or wound healing (type 2 EMT), but instead only undergo partial EMT (Aiello *et al.*, 2018) to reach a quasi-mesenchymal state (Dongre and Weinberg, 2019). Instead of epithelial protein expression being completely suppressed, these cells express proteins associated with both the epithelial and mesenchymal state. The resulting quasi-mesenchymal phenotype seems to confer greater cell motility to allow metastasis than the fully mesenchymal phenotype (Aiello *et al.*, 2018) and, of course, these cells also display sufficient plasticity to return to an epithelial state when the secondary tissue is penetrated. Formation of quasi-mesenchymal cells also leads to further difficulties associated with EMT and cancer treatment, as after transformation into this state, cells become resistant to both traditional chemotherapy and immunotherapy. Chemotherapy resistance to oxaliplatin- and cisplatin-based drugs has been shown in breast, ovarian, colon and pancreatic cancers, as a result of suppression of apoptosis by EMT-TFs, as well as through CSC formation (see later) (Lim *et al.*, 2013).

A key point here, is that cancer stem cells (CSCs) arise somewhere along the epithelial-to-mesenchymal transition pathway, although the exact molecular mechanisms remain to be elucidated (Dongre and Weinberg, 2019). The role of CSCs in the progression of the malignant phenotype are discussed later in this review.

Mechanisms involved in the early stages of EMT, such as the loss of cell adhesion, have been shown to be regulated by POPDC1. The dysregulation and loss of POPDC1 expression in cancer cells (e.g. CRC and HCC) have been shown to promote EMT in several studies (Williams *et al.* 2011; Han *et al.*, 2014; Han *et al.*, 2019). Studies by Han *et al.* highlighted a correlation between POPDC1 loss, enhanced cell motility and invasion, as well as a decrease in E-cadherin expression and an increase in SNAIL1, TWIST (EMT-TFs) and vimentin (a mesenchymal cell marker). Increased levels of vimentin and acquisition of a mesenchymal phenotype in POPDC1 knock-out cells was also observed by Kawaguchi *et al.* (2008). This is in accordance with the expression pattern associated with EMT induction described by Dongre and Weinberg (2019). Restoration of POPDC1 expression in colorectal cancer cell lines re-introduced an epithelial-like morphology (Williams *et al.*, 2014), further evidencing the fact that POPDC1 seems to play a key role in EMT switching.

3.3. Other factors influencing metastasis

VAMP3

VAMP3 is a vesicular transport protein (SNARE protein) that aids cell migration by inserting transferrin and β 1-integrin into the cell membrane (Parang *et al.*, 2018). POPDC1 proteins interact with VAMP3 via their C-terminal domain, which experiments have mapped to a region after aa118 (Hager *et al.*, 2010). As previously discussed, high levels of β 1-integrin proteins in the cell membrane are associated with the mesenchymal cell type (Dongre and Weinberg, 2019).

The POPDC1/VAMP3 interaction creates an equilibrium between β 1-integrin integration and recycling in the cell membrane to maintain cell function. If POPDC1 expression is suppressed in cancer cells, this interaction is lost, and VAMP3-mediated recycling of β 1-integrin is impaired. A disruption in this equilibrium leads to higher concentrations of β 1-integrin in the cell membrane which promotes migration.

Preventing the loss of POPDC1 proteins in cancer cells to maintain its interaction with VAMP3 may potentially provide a means to reduce cancer cell migration.

GEFT/Rac1/Cdc42

Furthermore, as described previously, POPDC1 has been shown to negatively regulate GEFT (Smith *et al.*, 2008), which in turn leads to reduced activation of Rac1 and Cdc42. These are members of the Rho family of GTPases and have been shown to induce membrane ruffling and lamellipodia, as well as filopodia and formation of actin microspikes, respectively (Guo *et al.*, 2003). These processes increase cell motility and promote cancer metastasis. Moreover, Rac1 and Cdc42 increase the transcriptional activities of serum response elements (SREs) (e.g. Elk1 and SAP1) of the c-fos proto-oncogene, which promote proliferation and induce EMT. As increased GEFT activity has been shown to induce lamellipodia, filopodia and actin cytoskeleton rearrangement through Rac1 and Cdc42 activity (Guo *et al.*, 2003), preventing this activity by inhibiting the POPDC1/GEFT interaction may be beneficial to reduce cancer cell metastasis.

Overactivation of Rac1 and Cdc42 signalling was also shown to decrease p53 (a well-known tumour suppressor) and increase VEGF expression in breast cancer cells (Ma *et al.*, 2013). This induces angiogenesis, another well-characterised hallmark of cancer (Hanahan and Weinberg, 2011). Increased Rac1 and Cdc42 levels in BC cells also correlated with increased metastasis and invasion (Ma *et al.*, 2013).

Despite Guo *et al.* (2003) showing that GEFT preferentially activates Rac1 and Cdc42 over RhoA, RhoA can be activated via other mechanisms, such as TJ downstream signalling pathways and the TJ-associated GEFH (Russ *et al.*, 2011). RhoA activates ROCK, which in turn stimulates myosin light chain kinase (MLCK), causing cytoskeletal rearrangement and allowing cell migration (Williams *et al.*, 2011). GEFH is usually sequestered to the TJ (via POPDC1 interaction) where it cannot activate RhoA (Parang *et al.*, 2018; Han *et al.*, 2015). Downregulation of POPDC1 and loss of TJ formation in cancer cells results in increased RhoA activation through the mechanisms outlined above, thereby triggering migration.

Studies by Wang *et al.* (2014) have identified that miRNA-122 is under-expressed in HCC cells. Besides leading to downregulation of POPDC1 (discussed previously), their studies also showed miRNA-122 to increase RhoA signalling and subsequently induce EMT. Restoring miRNA-122 expression attenuated RhoA signalling, promoted MET, and led to reduced cellular migration (Wang *et al.*, 2014). Hence, inhibiting the loss of miRNA-122 expression in cancer cells could therefore not only prevent metastasis by stabilising POPDC1 and maintaining the integrity of TJs, but also by dampening down RhoA-induced EMT.

Activation of the non-canonical Wnt signalling pathway has been shown to activate both Rac1 and RhoA (Zhan *et al.*, 2017). Wnt ligands activate the ROR-Fzd receptor complex which recruits Dvl proteins that polymerise at the cell membrane, or via activation of the membrane-bound Wnt5a receptor (see 2.4.). The former switches on RhoA and Rac1 signalling, which activates the ROCK signalling cascade, whilst the latter activation of the G_q-coupled Wnt5a receptor triggers an increase in intracellular Ca²⁺ concentration (Zhan *et al.*, 2017). All of the above trigger changes in actin polymerisation and cytoskeletal rearrangement, facilitating cell motility and metastasis.

In summary, combined activation of Wnt-signalling, RhoA, Rac1 and Cdc42 promotes EMT, migration, invasion, angiogenesis, as well as cell proliferation (discussed previously). Hence, inhibiting GEFT, Cdc42, Rac1, RhoA and Wnt signal activation, is a promising approach in the treatment of cancer and will be outlined in the outlook below.

NDRG4

Studies by Benesh *et al.* (2013) have shown that NDRG4 (n-Myc downstream regulated gene 4) binds to the CTD of POPDC1 (outside of the Popeye domain in the region of aa300-aa357). This binding site is unique to the POPDC1 protein and is not found in the other cAMP binding proteins. NDRG4 is a known tumour suppressor (Amunjela *et al.*, 2019), which represses cell proliferation, angiogenesis, metastasis, process extension and invasion, and like POPDC1 has been shown to also be downregulated due to hypermethylation in cancer cells (Benesh *et al.*, 2013).

The interaction between POPDC1 and NDRG4 has previously been identified in epicardial cells. A loss of this interaction resulted in impaired fibronectin trafficking, prevention of VAMP3 docking to the cell membrane and uncoordinated, non-directional cellular migration (Benesh *et al.*, 2013). While evidence for this interaction in cancer cells has yet to be established, stabilising the POPDC1 protein to maintain POPDC1-NDRG4 interaction could be a useful approach to prevent the spread of cancer cells.

Netrin-1

Netrin-1 is considered an oncogene, which inhibits apoptosis and promotes proliferation, differentiation, EMT and metastasis (Han *et al.*, 2015). Netrin-1 binds to four different receptors: deleted in colorectal cancer (DCC) and UNC5A, UNC5B and UNC5C. Netrin-1 is found to be upregulated in many cancer types, especially when the tumours are high grade or metastatic, such as BC, LC, CRC, PAC, GC and glioblastoma (Kefeli *et al.*, 2017). Of the four netrin-1 receptors, DCC and UNC5B have been shown to function as tumour suppressors in the absence of their ligand (Kefeli *et al.*, 2017).

The absence of netrin-1 allows DCC-mediated apoptosis through caspase activation, and the UNC5B receptor contains a binding site for p53 and enables p53-induced apoptosis. However, along with p53, the DCC and UNC5B receptors have been shown to be downregulated in CRC and GC through gene methylation (Kefeli *et al.*, 2017). Despite metastasis allowing the receptors to increase their distance from the netrin-1 ligand, their downregulation stops them from inducing apoptosis to prevent this migratory step (Arakawa, 2004).

Netrin-1 also promotes other cancer hallmarks, for example angiogenesis, by exerting a similar effect on the endothelium as VEGF (Kefeli *et al.*, 2017). It also influences proliferation and invasion, which have been shown to be mediated through DCC binding (Arakawa, 2004) and activation of the RhoA, Rac1, Cdc42 and PI3K signalling pathways (Ylivinkka *et al.*, 2016). Clearly, while netrin-1 promotes activation of these pathways, POPDC1 mediates inhibition. Therefore, netrin-1 up-regulation and corresponding POPDC1 downregulation is associated with a progressively more malignant phenotype, with both linked to downstream increases in RhoA, Rac1 and Cdc42 signalling in cancer cells.

Further evidence for netrin-1 promoting metastasis, invasion, and angiogenesis via PI3K/AKT and ERK-signalling was demonstrated by Zhang *et al.* (2018) in NSLCC, whilst Han *et al.* (2015) showed this to occur in HCC and correlate with POPDC1 downregulation. Subsequent re-expression of POPDC1 reduced the metastatic potential.

It has also been identified that netrin-1 upregulates EGF expression. The EGFR signalling pathway has been separately shown to decrease POPDC1 expression (Amunjela and Tucker, 2017a). It is therefore a reasonable assumption, that restoring POPDC1 expression and/or preventing its downregulation by netrin-1, could provide a means of preventing cancer metastasis.

3.4 Outlook - Maintaining Cell Adhesion and Inhibiting Metastasis by Stabilising POPDC1

From the data outlined above, limiting POPDC1 loss could prevent disassembly of tight junctions and loss of cell adhesion, metastasis and cell spreading, as well as abrogating POPDC1-loss-induced EMT. This could be achieved in three different ways: stopping POPDC1 downregulation through miRNA-122 loss or increased netrin-1 signalling, increasing POPDC1 expression, or by directly stabilising existing POPDC1 proteins. The latter may, for example, reduce changes in cell migration if its interaction with NDRG4 and VAMP3 to promote recycling of β 1-integrin were maintained.

The important role played by the RhoGTPase family in cancer has been discussed in detail above and highlights that this protein family presents itself as an ideal candidate for targeted cancer therapy. As the globular structure of the RhoGTPases has been deemed “undruggable”, alternative approaches need to be investigated (del Mar Maldonado and Dharmawardhane, 2018). Various small molecule inhibitors that target different parts of the RhoGTPase signalling pathway have already been developed or are currently undergoing clinical trials (Lin and Zheng, 2015). The most promising compounds act by disrupting the Rho-GEF interaction to inhibit RhoGTPase activation. It has been demonstrated that these approaches enhance the tumour response to classic anti-cancer agents such as cisplatin (e.g. in lung, renal cell and oesophageal squamous carcinoma) or EGFR/HER2 therapy in breast cancer (Clayton and Ridley, 2020; Zhang *et al.*, 2020; del Mar Maldonado and Dharmawardhane, 2018). Moreover, Rho-GEF inhibitors have been shown to reduce progression of some cancers (e.g. BC, leukaemia, melanoma, fibrosarcoma after EHOp-016 treatment) and various

specific inhibitors are already available for treatment (e.g. NSC23766, TBOPP and EHOp-016 (Rac1-specific), CASIN (Cdc42-specific) and LARG (RhoA-specific)) (Lin and Zheng, 2015; Clayton and Ridley, 2020; Tajiri *et al.*, 2017).

As Rho-GEF interaction inhibitors have shown promise in cancer drug development (del Mar Maldonado and Dharmawardhane, 2018), stabilising the POPDC1 protein to maintain its negative regulation of GEFT and inhibition of RhoGTPase activation may be a novel approach in the development of new anti-cancer treatments. While RhoGTPase proteins can be activated by numerous GEFs, not solely GEFT, inhibiting this one interaction may at least be a start in reducing Rac1, Cdc42 and RhoA signalling in cancer cells. When combined with the other corrections this stabilisation may achieve across other malignant cascades, this could prove a highly effective approach. Limited success has been demonstrated when the Rho/GEFT pathway is targeted by preventing Rho-effector protein interaction. Therefore, targeting POPDC1 stabilisation, may present itself as a viable option for reducing Rho/GEFT activation and enhancing POPDC1 presence, thereby maintaining its tumour suppressive activity.

To summarise, stabilising the POPDC1 protein in cancer cells could prevent metastasis, one of the main causes of cancer-associated deaths. First, however, more evidence for an interaction between POPDC1 and these proteins in cancer cells is required before these approaches can be further explored.

4. POPDC1 and Cancer Stem Cells – Relationship by Association

A malignant neoplasm is a heterogenous tissue consisting of many different cell types, including cancer-associated fibroblasts, cancer cells, epithelial cells, pericytes and inflammatory immune cells (Hanahan and Weinberg, 2011), which are involved in different aspects driving tumour progression. One of these cell types can self-renew and differentiate into all types of cancer cells (pluripotency) (Du *et al.*, 2019). These cells, known as cancer stem cells (CSCs), are implicated in the main difficulties of therapeutic cancer treatment. The formation of CSCs and quasi-mesenchymal cells somewhere along the EMT axis has already been discussed in detail, and this process is key to understanding anti-cancer drug resistance. CSCs are resistant to traditional cytotoxic chemotherapy agents due to their high expression of extrusion pumps (e.g. p-glycoprotein) (Zhang *et al.*, 2017; Shibue and Weinberg, 2017) and, similar to quasi-mesenchymal cells, possess mechanisms to avoid immune system detection. Furthermore, CSCs can also gain motility and undergo the process of metastasis outlined above and lie quiescent at local or distal sites for many years, before eventually re-entering the cell cycle leading to the development of a secondary tumour.

Anti-cancer drugs that specifically target CSCs could potentially solve these key issues driving disease progression (Zhang *et al.*, 2017), which perhaps makes CSCs the most promising target of all for developing new anti-cancer treatments (Yang *et al.*, 2020).

Signalling Proteins in CSCs

It has been shown that CSCs use the same signalling pathways as normal stem cells (Yang *et al.*, 2020). These include the Hedgehog, NF- κ B, JAK-STAT and PI3K/AKT/mTOR pathways and the Wnt pathway (Yang *et al.*, 2020). The latter is a critical signalling pathway in CSCs, and aberrant Wnt signalling has

been demonstrated in various leukaemias, BC, CRC, lung, prostate and ovarian cancer (Zhan *et al.*, 2017). A first generation of extracellular inhibitors of Wnt signalling have already entered clinical trials, e.g. vantictumab, a monoclonal antibody that inhibits the frizzled receptor (Du *et al.*, 2019).

The Wnt signalling pathway regulates multiple pathways that give CSCs their unique properties. For one, it ensures pluripotency and regulates CSCs differentiation. Furthermore, Zhan *et al.* (2017) also demonstrated that the Wnt pathway increased β -catenin-mediated expression of the TERT gene. TERT, telomere reverse transcriptase, is an enzyme that prevents telomere shortening in CSCs, giving them replicative immortality, another important hallmark of malignancies (Hanahan and Weinberg, 2011).

Signalling molecules of the tumour environment have been shown to maintain CSC properties via activation of the canonical and non-canonical Wnt pathways in CRC and BC (Zhan *et al.*, 2017). Furthermore, in APC-deficient cancers (e.g. colon adenoma or CRC) the Wnt signalling pathway is constitutively active, because loss of the APC gene leads to an increase in GEF-mediated activation of Rac1, which in turn stimulates NF- κ B (Myant *et al.*, 2013). This co-activation which increases Wnt signalling not only promotes cell proliferation but has also been shown to initiate de-differentiation of normal intestinal cells into CSCs (Schwitalla *et al.*, 2012).

The interaction of the POPDC1 protein with GEFT, an activator of Rac1, has been extensively discussed above. If the interaction between POPDC1 and GEFT could be maintained in CSC, it could reduce the activation of Rac1 and Wnt signalling outlined in this section. Despite the possibility of Rac1 being activated by other GEFs, reducing its activation by GEFT could provide a means to at least dampen down Wnt signalling in CSCs. It has also been highlighted in this review that POPDC1 negatively regulates Wnt signalling through its interaction with the LRP6 receptor, suggesting that POPDC1 stabilisation may have dual benefit. In summary, POPDC1 stabilisation introduces a new therapeutic approach to inhibiting the Wnt pathway intracellularly, at a point of integration between multiple signalling pathways.

Transcriptional Regulation in CSCs

The most important transcription factors in CSCs include Sox2, Nanog, Oct4, KLF4 and Myc (Yang *et al.*, 2020), of which the latter is involved in regulating cell metabolism, self-renewal, and proliferation. Its expression is often dysregulated in CSCs (Chen *et al.*, 2018), with high levels of c-Myc being achieved by reducing the amount of c-Myc ubiquitination and subsequent degradation, as well as increased Wnt signalling. c-Myc also promotes the hallmarks listed above and the formation of invasive CSCs.

As previously discussed, POPDC1 interacts with the regulatory PR61 α subunit of PP2A and increases the amount of c-Myc targeted for proteasomal degradation, as well as the Wnt signalling pathway to decrease β -catenin activation. Various experimental data supports that this PP2A-PR61 α interaction with the Wnt- and c-Myc-pathway regulates renewal and proliferation of stem cells (Thompson and Williams, 2018), yet a direct link to POPDC1 involvement remains to be established. However, if a link were confirmed, by association, one could hypothesise that POPDC1 may be key in regulating c-Myc expression by two different pathways (increasing PP2A activity and reducing Wnt signalling) in CSCs. Stabilising the POPDC1 protein to maintain this action may be able to reduce CSC proliferation by two separate mechanisms, making it a highly promising therapeutic target.

EMT and Differentiation

Two important steps in the metastatic cascade have been discussed in this review: the loss of cell adhesion which induces EMT, and the emergence of CSCs along the EMT axis, contributing to anti-cancer drug resistance and the formation of secondary tumours previously mentioned (Dongre and Weinberg, 2019).

POPDC1 downregulation has been shown to result in loss of cell adhesion and EMT (Williams *et al.*, 2011), and EMT-mediated induction of stemness has been evidenced in mammary carcinoma cells (Mani *et al.*, 2008) and intestinal stem cells (Reddy *et al.*, 2016). Although a link has yet to be established, the loss of POPDC1 inducing TJ disassembly and EMT could be driving the generation of CSCs. Thus, POPDC1 membrane stabilisation in cancer cells could potentially prevent EMT-induced formation of CSCs.

Studies by Reddy *et al.* (2016) on intestinal stem cells (ISC) showed that POPDC1 is downregulated after radiotherapy treatment. This correlated with an increase in Wnt signalling, evidenced by an increase in Wnt ligand concentration, frizzled receptor activation and downstream target gene transcription, which are key to ISC proliferation. Subsequent, rapid expansion of the ISC population was also observed, leading to the conclusion that POPDC1 downregulation and ensuing stem cell proliferation in response to radiation therapy, may be an underlying cause of resistance to radiotherapy treatment. The same phenomenon has also been observed as a result of POPDC3 downregulation in other cancers such as HNSCC, lung cancer and oesophageal cancer (Table 1) (He *et al.*, 2019). Stabilising these two isoforms of POPDC1 in the respective cancers may therefore reduce CSC formation and resistance to radiotherapy.

In addition, NF- κ B-induced (which is activated by Rac1) increases Wnt signalling in intestinal epithelial cells and has also shown to promote dedifferentiation of normal epithelial cells into tumour-initiating stem cells (Schwitalla *et al.*, 2012). One could hypothesise that the POPDC1 protein could be involved in this Rac1/NF- κ B co-activation of the Wnt signalling pathway as described above and that preventing POPDC1 loss in intestinal epithelia could result in reduced cell dedifferentiation into tumour-initiating CSCs.

The presence of POPDC1 proteins in CSCs and possible interaction with CSC signalling remains a relationship by association and still needs to be demonstrated experimentally. However, evidence shows that POPDC1 proteins play a crucial role in regulating c-Myc and Wnt-signalling pathways in normal cancer cells, and it is also known that CSCs use these exact same signalling mechanisms (Yang *et al.*, 2020; Dongre and Weinberg, 2019). If POPDC1 protein expression is also downregulated in CSCs, then stabilising the POPDC1 protein could be a useful therapeutic approach to directly inhibit cell proliferation and metastasis in CSCs.

5. Concluding Remarks

The many ways in which POPDC1 acts as a tumour suppressor in cancer cells by mediating cell adhesion and suppressing proliferation and metastasis through numerous signalling pathways have been discussed (Figure 2). However, its tumour-suppressive actions are often lost due to

downregulation in various types of cancer, including HCC, CRC, PC, BC, NSCLC, HNSCC, GC and glioma (Table 3).

Many downstream targets of POPDC1, such as ZO-1, GEFT/GEFH, Wnt-signalling, c-Myc, VAMP3 and netrin-1, are already established targets for drug development, however their ubiquitous expression may produce wide-ranging side effects. Hence, specifically stabilising POPDC1 in cancer cells to maintain interaction with these effectors may present itself as a novel, targeted approach to developing anticancer drugs. In line with the data presented above, POPDC1 stabilisation could have broad impact by making subtle changes to many different signalling pathways to promote cell adhesion, as well as reduce cancer cell proliferation and metastasis.

Future studies should aim to develop a small molecule that could stabilise POPDC1 and determine whether this would have the desired anti-tumour effects suggested above. Complete mapping of the interaction between POPDC1 and its target proteins to specific amino acid sequences will also be a vital step towards therapeutically targeting all POPDC1 downstream effectors. Further exploration of how POPDC1/PR61 α interaction may have a dual effect on both Wnt and c-Myc signalling may also be promising. Finally, investigation of whether POPDC1 is expressed in cancer stem cells (CSC) is crucial for confirming the possibilities of CSC targeting outlined in this review. The potential role POPDC1 may play in other hallmarks of cancer, such as angiogenesis, has been touched upon in this review and highlights the importance of this protein across a broad spectrum of characteristics of this disease. Further study may therefore unveil additional roles of POPDC1 in the progression of the malignant phenotype.

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7. Tables and Figures

Table 1

	POPDC2	POPDC3	Reference
Cardiovascular Disease	Under-expressed causing decreased conduction and AV block in cardiac muscle only. Associated with W188X mutation.	-	Brand, 2019 Amunjela <i>et al.</i> , 2019
Limb Girdle Muscular Dystrophy (LGMD)	-	Mutant (L155H, L217F, R261Q) POPDC3 expression leads to skeletal muscular dystrophy.	Vissing <i>et al.</i> , 2019
Ductal Breast Carcinoma (especially HER2+ subtype)	Over-expressed at all clinical stages. Possibly implicated in cancer initiation and sustenance.	Over-expressed at early clinical stages.	Amunjela and Tucker, 2017a
Head and Neck Squamous Cell Carcinoma (HNSCC)	-	Over-expression correlates with low patient survival. Potential biomarker for radiotherapy resistance.	He <i>et al.</i> , 2019
Gastric Cancer	-	Under-expression due to promoter hypermethylation. Lower POPDC3 levels correlate with increased depth of invasion and metastasis.	Lue <i>et al.</i> , 2012 Kim <i>et al.</i> , 2010
Oesophageal and Lung Cancer	-	Overexpression of POPDC3 correlates with greater radiotherapy resistance.	He <i>et al.</i> , 2019

Table 2

Protein	Interacting Sequence of POPDC1	Tissue location of POPDC1 interaction	Suggested Role	References
TREK-1	Unknown sequence on CTD	Cardiac myocytes	Interaction with POPDC1 enhances current flow in cardiac myocytes	Han <i>et al.</i> , 2019 Brand, 2019
CAV-3	aa242-266	Skeletal muscle sarcolemma, cardiac myocyte transverse tubules	POPDC1 ensures structural integrity and function of Cav-3	Han <i>et al.</i> , 2019 Brand, 2019
VAMP3	CTD sequence after aa118	MDCK cells, adult cardiac and skeletal muscle	POPDC1 interaction ensures adequate recycling of β 1-integrins. Loss of this interaction increases migration.	Hager <i>et al.</i> , 2010
GEFT	aa250-300	Human Corneal Epithelia, Murine NIH T3T Cells	Retention of GEFT in membrane, preventing Rac1/Cdc42/RhoA activation promoting TJ formation	Russ <i>et al.</i> , 2010, Smith <i>et al.</i> , 2008
GEFH	Unknown sequence on CTD	Human Corneal Epithelia	POPDC1 sequesters GEFH to cell membrane to prevent RhoA signalling	Russ <i>et al.</i> , 2011, Parang <i>et al.</i> , 2018
ZO-1	Unknown sequence on CTD	Trabecular meshwork cells, HCE, uveal melanoma	POPDC1/ZO-1 interaction prevents ZONAB-induced entry to cell cycle and translation of proliferative genes	Russ <i>et al.</i> , 2010, Russ <i>et al.</i> , 2011 Amunjela <i>et al.</i> , 2019, Jayagopal <i>et al.</i> , 2011
Occludin	Unknown sequence on CTD	HCE, uveal melanoma	Maintenance of tight junction formation	
Snip3	Unknown sequence on CTD	Cardiac Myocytes	POPDC1 suppresses Snip3-induced apoptosis	Kliminski <i>et al.</i> , 2016
LRP6 (Wnt/β-catenin-pathway)	Unknown sequence on CTD	HEK293 cells, human colonoids, murine adenoma tumoroids	Prevention of β -catenin activation by inhibition of LRP6	Thompson <i>et al.</i> , 2019
PR61α (c-Myc pathway)	aa330-345	Murine colitis-associated cancer cells	Promotes c-Myc ubiquitination / degradation	Parang <i>et al.</i> , 2017

Table 3

Mechanisms of POPDC1 Downregulation	Cancer type	Reference
Promoter Hypermethylation	CRC, PC, BC, NSCLC, Glioma, HNSCC, GC	Williams <i>et al.</i> , 2010; Parang <i>et al.</i> , 2017, Amunjela and Tucker, 2016; Kim <i>et al.</i> , 2010.
Under-expression of miRNA-122	HCC	Wang <i>et al.</i> , 2014
Over-expression of Netrin-1	HCC	Han <i>et al.</i> , 2015
EGFR activation	BC	Amunjela and Tucker, 2017a

Figure 1

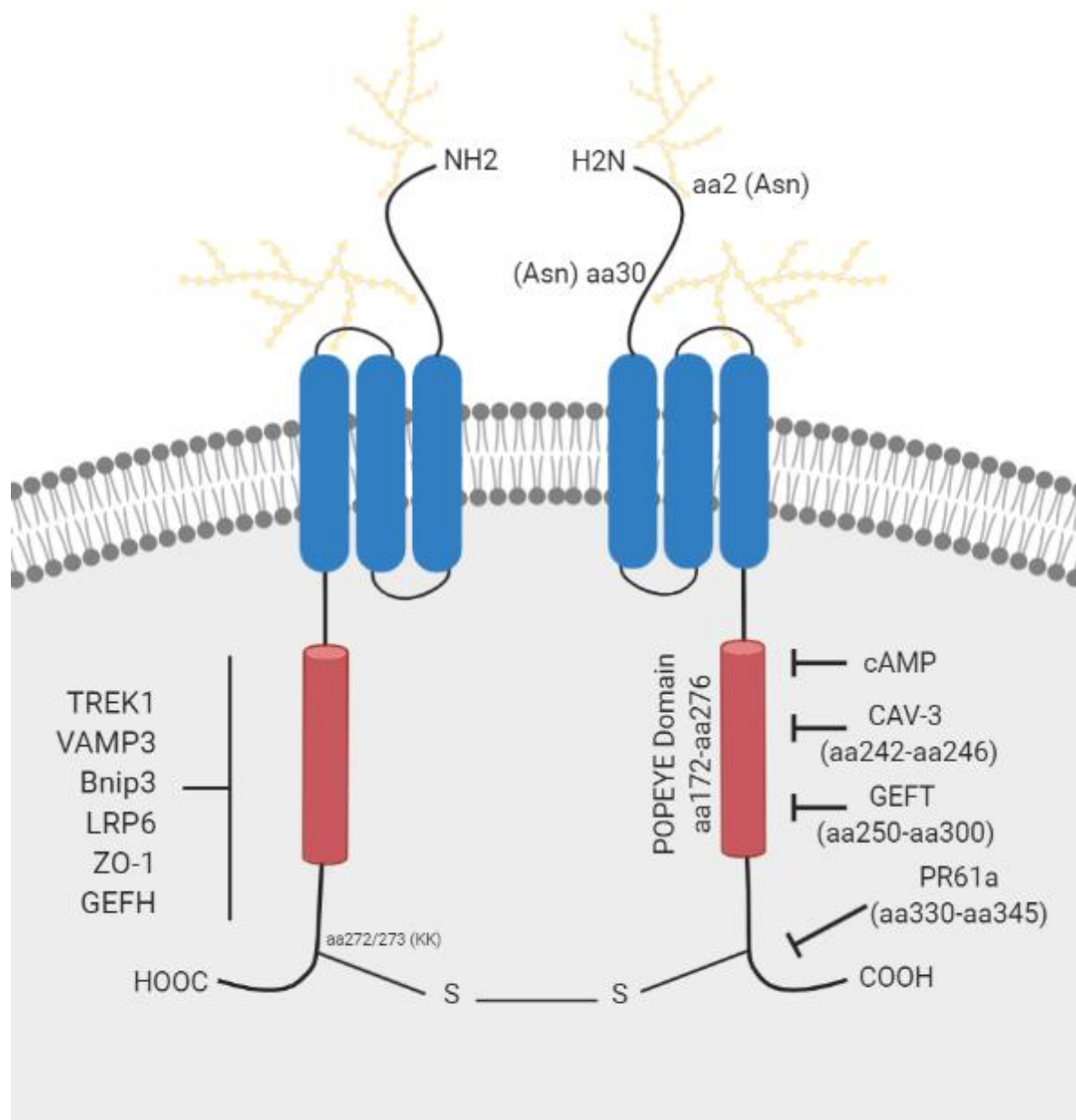


Figure 2

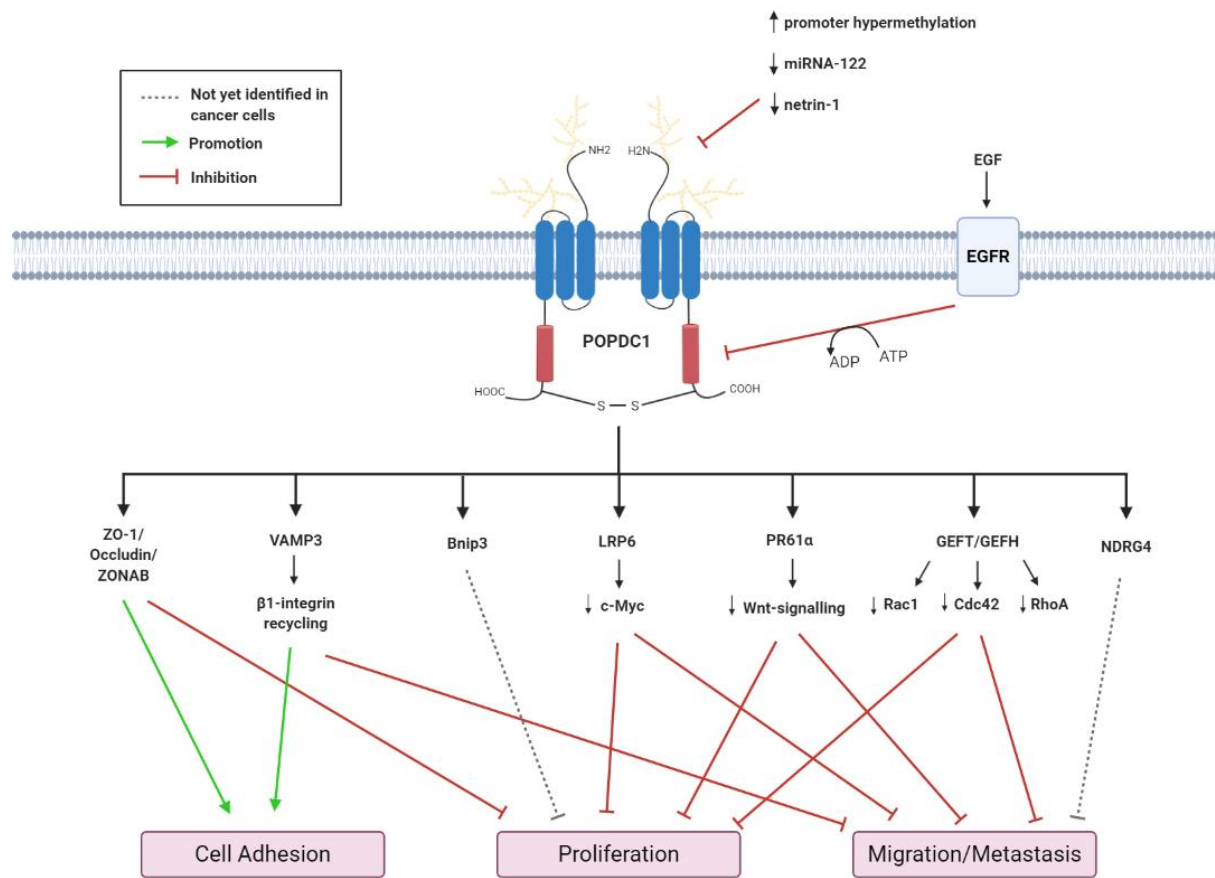


Table 1 - The Role of POPDC2 and POPDC3 in Disease

Expression of POPDC2 and POPDC3 varies between tissue type and across various cancer types. Dysregulation of POPDC2 is mainly observed in cardiovascular disease and breast cancer. POPDC3 mutations are implicated in limb girdle muscular dystrophy and has been shown to have both tumour-suppressive and oncogenic roles in different malignancies.

Table 2 – POPDC1 Downstream Targets

The POPDC1 protein interacts with many downstream including TREK1, CAV-3, VAMP3, GEFT, GEFH, ZO-1, occludin, Bnip3, LRP6 and PR61 α . This interaction has mainly been shown in cardiac and skeletal muscle cells, however, an increasing body of evidence is emerging that demonstrates POPDC1 interaction with these targets in cancer cells.

Abbreviations: CTD (C-terminal domain), HCE (human corneal epithelial cells), HEK293 (human embryonic kidney cells)

Table 3 – Mechanisms of POPDC1 Downregulation Associated With Various Cancer Types

The four main mechanisms of POPDC1 downregulation include promoter hypermethylation, under-expression of miRNA-122 reducing POPDC1 gene transcription, over-expression of netrin-1 leading to inhibited POPDC1 expression and EGFR activation, which phosphorylates and inactivates POPDC1. These mechanisms have been observed in many different cancer types including HCC, CRC, BC, PC, NSCLC, HNSCC and glioma. The most commonly identified mechanism of POPDC1 downregulation is promoter hypermethylation.

Abbreviations: CRC (colorectal cancer), PC (prostate cancer), BC (breast cancer), NSCLC (non-small cell lung cancer), HNSCC (head and neck squamous cell carcinoma), GC (gastric cancer), HCC (hepatocellular carcinoma).

Figure 1 – The Structure of Popeye Domain Containing Protein 1 (POPDC1)

POPDC1 is a transmembrane protein highly expressed in cardiac myocytes, skeletal muscle and some cancer cells. The short extracellular N-terminal domain contains two glycosylation sites (asparagine residues at positions aa2 and aa30). This is followed by three membrane-spanning domains and a long intracellular C-terminal domain (CTD). The CTD contains the highly conserved POPEYE domain (aa172-aa276) with a unique cAMP binding site. Other binding sites exist for TREK1, VAMP3, Bnip3, LRP6, ZO-1, GEFH, CAV-3 (aa242-246), GEFT (aa250-300) and PR61 α (aa330-aa345). A disulfide bridge forms between intracellular cysteine residues with lysine residues (aa272/273) critically required for this dimerisation, which promotes membrane stabilisation.

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Figure 2 - Regulatory Roles of POPDC1 in Cancer

POPDC1 acts as a tumour suppressor by influencing three main processes involved in cancer progression. Its interaction with tight junction-associated proteins (e.g. ZO-1, occludin, ZONAB) and VAMP3 ensures maintenance of cell adhesion. Furthermore, POPDC1 inhibits uncontrolled cell proliferation through its interactions with guanine nucleotide exchange factors (GEFT/GEFH) and the c-Myc and Wnt-signalling pathways. POPDC1 also plays an important role in suppressing cancer cell migration and metastasis.

Loss of these regulatory roles exerted by POPDC1 can occur as a result of intracellular phosphorylation (through EGFR) or reduced gene expression due to promoter hypermethylation, reduced miRNA-122 expression or increases netrin-1 activity.

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