Approaches to Investigating the Protein Interactome of PTEN

Sarah L. Smith[1], Andrew R. Pitt[1,2] and Corinne M. Spickett[1]*

[1] School of Life and Health Sciences, Aston Triangle, Aston University, B4 7ET, Birmingham, UK

[3] Manchester Institute of Biotechnology, University of Manchester, 131 Princess Street, Manchester. M1 7DN, UK

*** Corresponding author Email: c.m.spickett@aston.ac.uk**

ABSTRACT

The tumour suppressor phosphatase and tensin homologue (PTEN) is a redox-sensitive dual specificity phosphatase with an essential role in the negative regulation of the PI3K-AKT signalling pathway, affecting metabolic and cell survival processes. PTEN is commonly mutated in cancer and dysregulation in the metabolism of PIP_3 is implicated in other diseases such as diabetes. PTEN interactors are responsible for some functional roles of PTEN beyond the negative regulation of the PI3K pathway and are thus of great importance in cell biology. Both high-data content proteomics-based approaches and low-data content PPI approaches have been used to investigate the interactome of PTEN and elucidate further functions of PTEN. Whilst low-data content approaches rely on co-immunoprecipitation and western blotting, and as such require previously generated hypotheses, high-data content approaches such as affinity pull down proteomic assays or the yeast 2-hybrid system are hypothesis generating. This review provides an overview of the PTEN interactome, including redox effects, and critically appraises the methods and results of high-data content investigations into the global interactome of PTEN. The biological significance of findings from recent studies is discussed and illustrates the breadth of cellular functions of PTEN that can be discovered by these approaches.

KEY WORDS

Protein-protein interactions, global interactome, high-data content, affinity pull down, yeast 2-hybrid, redox regulation.

TEXT

1.0 Introduction

Phosphatase and tensin homologue (PTEN), also called mutated in multiple advanced cancers-1 (MMAC-1) and tensin-like phosphatase-1 (TEP-1), is a 403 amino acid protein first identified in 1997 by three independent research groups [1-3]. PTEN has been identified as an antagonist of the AKT pathway through lipid phosphatase activity on phosphatidylinositol-3,4,5-phosphate (PIP_3) to form phosphatidylinositol-4,5-phosphate (PIP_2), thus reversing the effect of phosphatidylinositol-3-kinase (PI3K) [4]. Negative regulation of the AKT pathway, through conversion of PIP_3 to PIP_2, modulates several metabolic processes, from lipogenesis to glycolysis as well as cell growth, proliferation and apoptosis [5]. In this way, PTEN activity determines important aspects of the cellular metabolic landscape, including dysfunction in a variety of diseases. PTEN is regulated both by post-translational modification (PTM), including phosphorylation and oxidation, and through important interactions with proteins such as protein kinase casein kinase 2 (CK2) and thioredoxin (Trx), highlighting the key importance of uncovering PTEN's interactome [6, 7].

Within the cell, large protein networks are composed of both highly and poorly connected systems that receive inputs and generate outputs [8]. Signal transduction is an essential cellular process, composed of sequential and controlled protein-protein interactions to modulate cellular processes, mediated by protein-binding domains [9]. Protein-protein interactions have notable effects, an example of which is activation/inactivation of enzymes through PTMs such as phosphorylation and oxidation, which can alter protein conformation, catalytic activity, and downstream interactions [10-12]. It is now widely accepted that a range of physiological and pathophysiological states alter protein-protein interactions (PPIs) of signalling proteins [13, 14].

Advances in high-data content techniques for interactor identification, using both *in vitro* approaches, such as affinity pull-down assays, and *in cellulo* approaches, such as the yeast-2-hybrid (Y2H) assay, have allowed progress in mapping PPIs of many signalling proteins, including PTEN. Comparisons of these two high-data content techniques noted relatively limited levels of PPI overlap between the different approaches [8]. This may result from high false positive and negative rates. In one study, affinity purification and Y2H were estimated to have 40-80% false negative and 30-60% false positive rates [15], while another on Y2H reported a 25-45% false positive and a 75-95% false negative rate [16]. During a comparison of models of bait-prey interactions, a 39% false positive rate for affinity chromatography was described [17]. This issue is highlighted by the comparison of proteins identified as part of the yeast proteome, where 80,000 interactions are identified but only 3% (2,400) of these are supported by more than one method [15]. In addition, comparisons of independent Y2H screens showed <30% overlap due to the small fraction of the interactome detected per screen [18, 19]. High false discovery rates continue to be an issue, with more recent papers focussing on the source and reduction of false positive PPIs [20-25]. However, it has been concluded that both techniques produce high-quality data that are complementary in nature [26]. The limitations of both techniques, such as false positive hits, can in part be counteracted through the use of 'dry' and 'wet' techniques to validate a much smaller subset of the interactors identified. 'Wet' techniques include co-immunoprecipitation and western blotting through to affinity kinetic experiments such as surface plasmon resonance (SPR). 'Dry' techniques usually involve statistical and bioinformatics approaches where a confidence score is applied to identifying potential interacting proteins based on similarity in areas such as expression profile, function and sequence homology [27]. The use of co-immunoprecipitation and western blotting can also form the basis of low-data content investigations into singular protein-protein interactions. A cycle of high-

data content hypothesis generation and low-data content hypothesis testing acts to strengthen interactome data sets, whilst also providing information on the function and wider implications of the protein-protein interaction.

While there are many excellent reviews on the structure, function and regulation of PTEN, as yet none have focused specifically on analysis of the approaches used to investigate its interactome, and the effect of modification and mutation on protein-protein interactions. The aim of this review is to bring together information on the different approaches that have been used to study PTEN interactors and the information obtained from them.

1.1 Highlights

- PTEN's membrane recruitment and phosphatase activity are regulated by PPIs.
- High-data density methods such as Y2H and affinity pull down assays have been utilised successfully to identify protein interactors of PTEN.
- Identified interactors have been validated using techniques such as co-immunoprecipitation and western blotting.
- Functional validation of a small subset of interactors has been elucidated using *in vitro* and *in situ* assays.
- Mutation and oxidation of PTEN inhibits phosphatase activity and alters PPIs.

2.0 Structure, Function and Regulation of PTEN

2.1 The Structure and Function of PTEN

In order to appreciate the PPIs of PTEN, in particular the sites of interaction within PTEN and their effects on its function, it is essential to have an overview of its structure; this is also important

for the design of proteomic approaches to investigate PPIs. PTEN has two major domains, an N-terminal phosphatase domain and a C-terminal membrane binding domain [28]. The N-terminal phosphatase domain shows homology to the dual-specificity phosphatase (DUP) *Vaccinia* H1-related (VHR) with conserved stretches of amino acids in the phosphate binding (P) loop, the TI loop and the WPD loop [29] (Figure 1) which are all essential for catalysis and conformation [28]. The P loop contains the protein tyrosine phosphatase (PTP) conserved HCXXGXXR motif essential for phosphatase activity, whereby the catalytic cysteine (C124) acts as a nucleophile through which the phosphoryl group is transferred, forming a thiol phosphate intermediate during catalysis [30-34]. The WPD-loop, which contains an FED (F90-E91-D92) motif, is also called the movable loop due to its ability to move into the active site on substrate binding (known as a closed conformation) [34]. This movement allows D92 to participate in the hydrolysis of the cysteine thiol phosphate intermediate [32, 34]. The key difference between the active site of PTEN and other PTPs is the increased width of the active site pocket, owing to a 4-amino acid insertion to the TI loop [28]. This insertion allows accommodation of PTEN's physiological substrate phosphoinositide-3,4,5-phosphate (PIP_3), which is larger than those of most other PTPs and DUPs [28]. Recent evidence has shown residues T167 and I168 to be essential for substrate specificity [35].

The C-terminal domain can be further subdivided into the C2 domain, the C-terminal tail and the PDZ motif. The C2 domain is a calcium binding domain which permits binding to phospholipids, inositol polyphosphates and phosphotyrosine domains (PTB), allowing the association of PTEN and the cell membrane [28, 36, 37]. The C2 domain has extensive contact with the phosphatase domain and so assists PTENs catalytic activity through a productive orientation of the active site at the membrane [28, 38, 39]. The C-terminal tail is an intrinsically disordered region (IDR) that contains a number of binding motifs and sites of post-translational modification, and is

a key part of the protein for mediating PPIs [40]. Aberrant post-translational modification or mutation of this region may be important in disease, and it may also, therefore, present a new drug target. This is discussed further in the sections below. The C-terminal tail contains proline/serine/threonine/glutamic acid rich (PEST) sequences related to half-life and stability [41, 42]. A region of inhibition through phosphorylation is found in the C-terminal tail which includes residues T366, S370, S380, T382, T383 and S385 [43-45]. Phosphorylation of the C-terminal tail reduces both the phosphatase activity as well as PTEN's lipid membrane affinity through a conformational change, whereby the tail interacts with the C2 domain [45]. This closed conformation blocks membrane binding as well as the active site [46]. Auto-dephosphorylation removes the phosphates from T366 and S370 to allow phosphatase activity [46, 47]. The three terminal residues of the C-terminal domain, T401, L402 and V403, form the PDZ-motif, a typical module where protein-protein interactions can occur [28, 48-51].

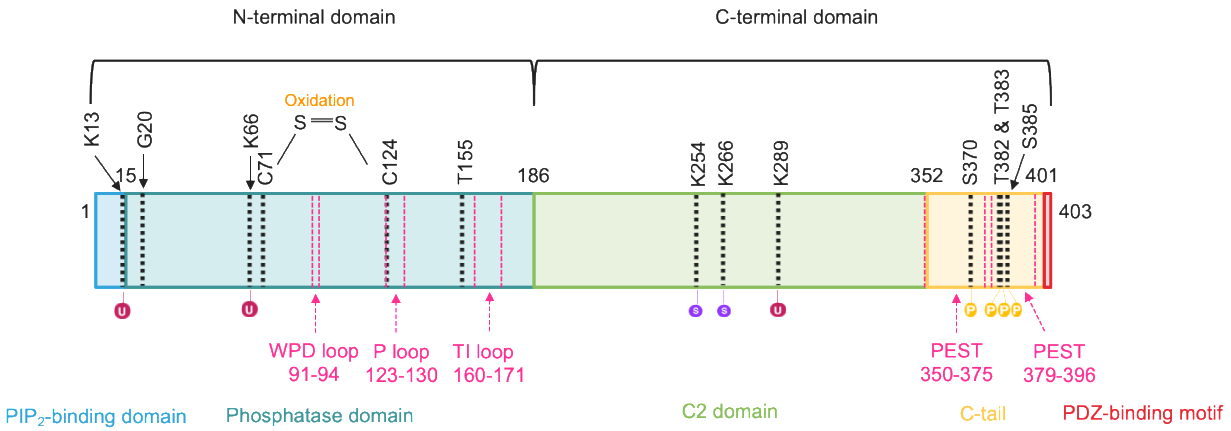

Figure 1. The N- and C- terminal domains of PTEN. The modular structure of PTEN shows N-terminal and C-terminal domains. The N-terminal PIP_2 binding domain (blue) contains K13, which can be modified by ubiquitinylation (U). The phosphatase domain (dark green) contains the WPD loop, P loop and TI loop. Within the phosphatase domain is the resolving C71 and catalytic C124,

which on oxidation form a disulfide bond. Two residues, G20 and T55, are mutated in two of the high-data content studies included in this review. The C2 domain (green) contains K254, K266 and K289 which are amino acids modified by ubiquitinylation (U) and SUMOylation (S). The C-terminal tail (yellow) contains two PEST sequences, as well as four residues which can be modified by phosphorylation (P); S370, T382, T383 and S385. The PDZ-binding motif is shown in red.

2.2 Isoforms of PTEN

Two extended isoforms of PTEN have been identified: PTENα (or PTEN-long, PTEN-L) with an additional 173 N-terminal amino acids, and PTENβ with an additional 146 N-terminal amino acids [52-54]. In comparison to canonical PTEN, these translational isoforms vary in their subcellular location.

PTENα is localised in mitochondria, with involvement in the maintenance of mitochondrial structure and function, including the promotion of energy production [52]. Recently, PTENα has been reported as a protein phosphatase for ubiquitin, acting to inhibit mitophagy [55], the selective autophagy to remove damaged and impaired mitochondria where there is loss of inner mitochondrial membrane potential that is essential for energy production [56, 57]. This has important implications in Parkinson's disease, the pathogenesis of which is linked to defective mitophagy [55]. A further study reported PTENα as a membrane permeable and secreted form of PTEN [53]. As an exogenous agent, PTENα was found to be able to enter other cells and downregulate PI3K signalling in the same manner as canonical PTEN [53].

PTENβ is found in the nucleolus, associated with the phosphoprotein nucleolin [54]. Dephosphorylation of nucleolin by PTENβ downregulates ribosomal deoxyribonucleic acid (rDNA) transcription and, therefore, ribosomal biogenesis and cell proliferation [54]. Nucleolin

upregulates cell proliferation and through the identification of increased growth in PTENβ knockout cells, PTENβ has been proposed to control cell proliferation through the downregulation of rDNA synthesis [54]. A recent study reported that PTENα and PTENβ promote tumorigenesis through maintenance of cancer promoting signature [58]. Whilst this review focuses on canonical PTEN, it is important to note that differences would be expected in the interactomes of the different PTEN isoforms, not only due to the N-terminal extensions and the additional corresponding binding domains, but also differences in extra- and subcellular locations.

2.3 PTEN as a Tumour Suppressor and a Metabolic Regulator

The biological functions of PTEN are diverse and have been reviewed previously [59-61]. Its role in signalling pathways provides an initial pointer to its likely interactors, and helps to inform the choice of proteomics approaches to investigate them. PTEN was initially identified as a tumour suppressor through demonstration of its role as a down-regulator of AKT signalling pathways through reduction of the cellular levels of PIP_3 [4]. Recruitment of AKT to the cell membrane occurs through its pleckstrin homology domain (PH), which binds to PIP_3 [62]. This binding allows the activation of AKT through phosphorylation at T308, a catalytic T-loop residue, by phosphoinositide-dependent protein kinase 1 (PDK1) [63]. PDK1 is recruited through binding via its PH domain to PIP_3 [64]. Maximal activity of AKT is seen when S473, a C-terminal residue, is phosphorylated by mammalian target of rapamycin complex 2 (mTORC2) preserving the activation state of AKT through stabilisation of T308 phosphorylation [65-67]. mTORC2 is activated by phosphorylated AKT (pAKT), giving a positive feedback loop [66]. The activation of AKT and its regulation by PTEN have a wide variety of downstream effects from cell survival to metabolism (Figure 2) [68-70].

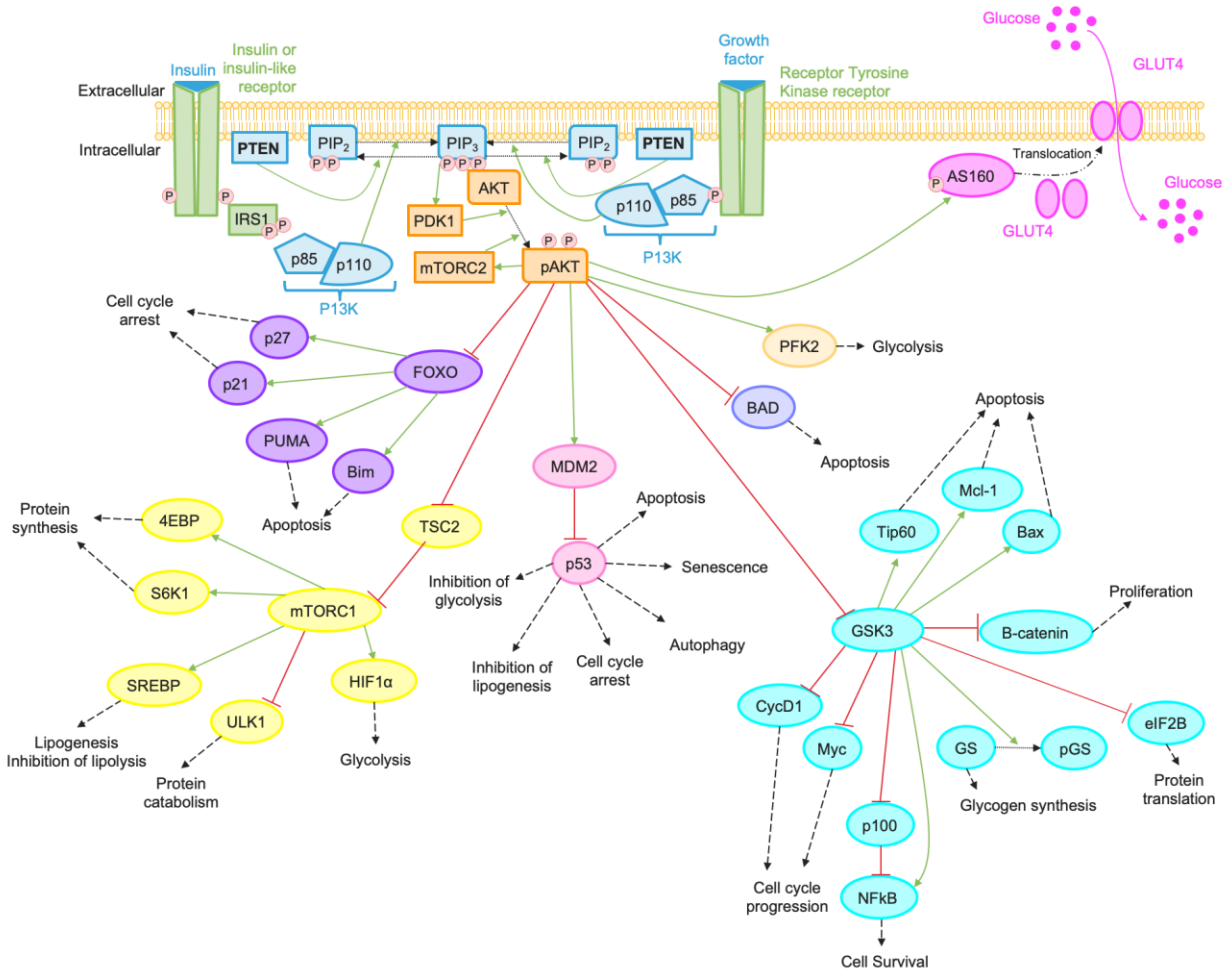

Figure 2. Schematic of PTEN's role in and the downstream effects of the AKT pathway. The pathway is activated at the cell membrane; downstream, phosphorylated AKT signals to FOXO, MDM2, GSK3 and BAD nodes to regulate cell survival versus cell death, while TSC2, mTORC1, PFK2 and AS160 nodes have more impact on metabolism.

Table 1. The effect of an increase in PIP_3 on metabolism and cell survival

Metabolic effects:	**Cell survival effects:**

1. Protein synthesis 2. Lipogenesis 3. Inhibition of lipolysis 4. Glycogen synthesis 5. Glucose uptake 6. Glycolysis 7. Suppression of protein catabolism (autophagy)	1. Cell cycle progression & inhibition of cell cycle arrest 2. Cell survival & inhibition of apoptosis 3. Proliferation

Figure 2 outlines how the PI3K-PTEN regulatory balance plays an important role in cell survival and proliferation via control of AKT signalling and the linked regulatory nodes of GSK3, mTORC1, FOXO and p53 [71-80]. In addition to these large networks, AKT also mediates smaller networks, such the activation of PFK2 and AS160 [81-83] (Figure 2). As PTEN is the key regulator in the cellular levels of PIP_3, PTEN activity has many physiological consequences, summarized in Table 1. Activation of insulin receptors through binding of insulin and insulin-like growth factors results in the activation of signal transduction of pathways involved in the regulation of both glucose and lipid metabolism [5, 68]. Increased PIP_3 upregulates protein, glycogen and lipid synthesis as well as glycolysis and glucose uptake, whilst downregulating lipolysis and protein catabolism. Loss of regulation by PTEN leads to improved insulin sensitivity, reducing the risk of type 2 diabetes, although also increasing the risk of obesity [5, 84]. Activation of receptor tyrosine kinases (RTKs) with growth factors leads to recruitment of PTEN and its modulation of downstream effects regulating cell survival. These include cell cycle progression and inhibition of cell cycle arrest with inhibited apoptosis and an increase in cell survival and proliferation, highlighting the important tumour suppressive function of PTEN. Alterations in both cell survival mechanisms and

metabolic processes are essential for tumour progression [80]. Tumour suppression by PTEN is apparent from the effects of loss or mutation of PTEN leading to catalytic inhibition, which results in neoplastic disorders such as Cowden's syndrome [85, 86].

2.4 Regulation of PTEN's activity, interactions and cellular localization by post-translational modification

PTEN can be regulated through both genetic and non-genetic means, the mechanisms of which have previously been reviewed [87, 88]. Genetic regulation of PTEN includes genetic alterations, epigenetic silencing and transcriptional and post-transcriptional regulation [59, 89, 90]. Non-genetic regulation of PTEN's activity includes post-translational modification (PTM) such as phosphorylation, oxidation, reduction, nitrosylation, and ligation with ubiqituin and small ubiquitin like modifier (SUMO) [6, 7, 89, 91-93]. PTMs can also affect PPIs, as discussed in Section 2.5, and PPIs themselves can regulate PTEN [61, 88, 94].

Phosphorylation at the C-terminal tail by protein kinase CK2 prevents the recruitment of PTEN to a multimeric complex at the cell membrane through conformational changes acting to mask the PDZ-motif [6]. The resulting 'closed' structure of PTEN prevents access to PIP_3, removing PTEN's modulation of the AKT pathway [6, 95]. It has been shown in human T cell acute lymphoblastic leukemia cells that protein CK2 phosphorylation of PTEN leads to constitutive activation of the AKT pathway through stabilisation of PTEN's expression and inhibition of PTEN's catalytic activity [96]. The acidic nature of the amino acids flanking Y383, as part of PTEN's C-terminal tail, suggested similarities to PTEN's physiological substrates and led to a hypothesis that Y383 could be a site of auto-dephosphorylation [43, 97]. As protein kinase CK2 is constitutively active,

dephosphorylation of PTEN may be a key mechanism regulating its activity [6, 98], similarly to MAPK signalling pathways [99].

In addition to phosphorylation, oxidation of PTEN is an important mechanism of regulation and results in its inactivation [7]. Oxidation of the catalytic cysteine (C124) by oxidizing species such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) have been documented to cause dose- and time-dependent inactivation of PTEN [7, 100]. Whilst free cysteine residues in aqueous solution have a calculated pK_a of 8.6, due to the nature of the flanking amino acids the calculated pK_a of C124 is 4.5. This lower pK_a means the thiol group (-SH) exists as a thiolate ion ($-S^-$) at physiological pH [30, 101]; thiolate ions have increased reactivity, a property that is essential for nucleophilic catalysis, but also result in an increased susceptibility to oxidation [101-103]. On oxidant exposure, the thiolate ion is oxidised to sulfenic acid (-SOH), which is capable of rapidly forming disulfide bonds with proximal cysteines; in the case of PTEN this is the so called resolving cysteine, C71 [7]. The formation of this intramolecular disulfide bond inactivates PTEN, and as with other phosphatases is believed to protect it from further (over-)oxidation, which can be reversed by reduction with glutathione (GSH), dithiothreitol (DTT) or redox proteins such as thioredoxin (Trx) and glutaredoxin (Grdx), which all restore catalytic activity [7, 91, 104]. A further element to the redox regulation of PTEN includes S-nitrosylation of cysteine thiol groups by *S*-nitrosocysteine (CSNO), leading to the formation of a protein mixed disulfide and reversibly inhibiting PTEN's catalytic activity [105]. In addition, through the ubiquitin protease system (UPS), nitric oxide (NO) induces PTEN degradation [93].

The reversible ligation of ubiquitin and SUMO to PTEN controls not only protein stability, but also cellular localization [92, 106]. Ubiquitination of PTEN is mediated by several E3 ubiquitin ligases, including NEDD4-1 and WWP2 [107]. Notable sites of ubiquitination on PTEN include K13,

K66 and K289 [92, 108]. Polyubiquitination of PTEN is linked to degradation through the ubiquitin proteasome system (UPS), whilst monoubiquitination leads to nuclear import [92, 107, 109]. SUMOylation of PTEN at K254 and K266 in the C2 domain promotes binding to the cell membrane, facilitating PTEN activation and the downregulation of the AKT pathway [110]. SUMOylation of PTEN is also associated with nuclear retention and DNA repair during genotoxic stress [111, 112].

2.5 Protein-Protein Interactions with PTEN

The mechanisms by which the membrane binding, cellular location and catalytic activity of PTEN is regulated by PTMs, facilitated by or altering protein-protein interactions (PPIs), as described in section 2.4, highlights the importance of the interactome of PTEN. The role of PPIs in the regulation of PTEN has previously been reviewed [61, 88, 94], which illustrates the range of effects of PPIs. At the time of writing, BioGrid [113] reports 755 published interactions with PTEN (Supplementary table 1), corresponding to 551 unique interactors. The associated gene ontology (GO) biological processes of these interacting proteins encompasses many different processes including those involved in cell signalling, cell survival and immune response, as well as organ function and the central nervous system (Supplementary table 2) [113]. An additional interaction database, IntAct, describes 227 binary interactions with PTEN, by a total of 95 interactors (Supplementary table 3) [114]. IntAct is one of the partners of BioGRID and so would be expected to contain all of the interactions reported by BioGRID (Supplementary table 1). However, a small number of additional PTEN interactions reported by IntAct are not found within the BioGRID dataset.

2.6 Methods to Identify PTEN Interacting Proteins

High-data content investigations into protein-protein interactions generate hypotheses, allowing new directions and lines of enquiry to be discovered, helping to reveal previously unknown aspects of PTEN's interactions and function, In contrast, low-data content investigations are hypothesis-driven and are useful for providing detailed mechanistic information on individual PTEN interactions and validating high-data content output. Methods for low-data content, hypothesis-driven investigations include co-immunoprecipitation and western blotting, whilst methods for high-data content, hypothesis-generating investigations include affinity pull down assays and the yeast-2 hybrid assay. Co-immunoprecipitation and western blotting are also used in high-data content investigations as validation steps. Whilst the yeast-2 hybrid assay was originally a low-data content method used on a small scale for single proteins, this method has been scaled up to become high-throughput [115, 116].

Important examples of interactors studied by hypothesis driven, low-data content investigations into PTEN's interactome are thioredoxin (Trx), peroxiredoxin (Prdx-1) and DJ-1. As discussed in section 2.4, PTEN's phosphatase activity is inactivated by oxidation through intramolecular disulfide bond formation [7]. Two known cellular antioxidants, Trx and GSH, were tested for their ability to reduce this disulfide bond by monitoring PTEN mobility shifts induced by disulfide bond formation in non-reducing gels, with Trx identified as the more efficient PTEN reductant [7, 117]. It was noted that the significant change in mobility on formation of the disulphide suggests this induces some conformation change in the protein, as well as the potential change in charge state. There have since been further *in vitro* and *in vivo* investigations into the relationship of Trx and PTEN [118-121]. Prdx-1 is a peroxidase isoform with a role in redox regulation through reduction of oxidants such as hydrogen peroxide (H_2O_2) [122]. Increased PTEN oxidation was seen with loss of

Prdx-1 and an interaction was confirmed through western blotting [123]. Under mild oxidative stress, using a 25 μM H_2O_2 treatment, PTEN's lipid phosphatase activity for PIP_3 was protected by Prdx-1[123] . This was lost under conditions of higher oxidative stress, with a 500 μM H_2O_2 treatment, where there was decreased PTEN-Prdx-1 binding, which is further discussed in section 4.4 [123]. DJ-1 is an oncogenic protein with a role in tumourigenesis and cancer progression [124, 125]. DJ-1 was identified as a negative regulator of PTEN through the P13K pathway using *Drosophila* as a model for mammalian systems [126]. Additionally, transient transfection of PTEN and/or DJ-1 in mouse fibroblasts provided evidence that DJ-1 rescued the cells from PTEN-induced apoptosis [126]. More recently, this has been expanded to investigate whether activation of the AKT pathway was the cause of DJ-1's oncogenic effects [127]. Increased DJ-1 expression gave decreased PTEN levels resulting in accelerated colorectal cancer and as such, DJ-1 has been proposed as a novel biomarker for prognosis in colorectal cancer [127].

To date, there are only 8 published studies specifically investigating PTEN's interactome using eukaryotic, non-genetic and high-data content methods (Table 2). Each of these studies utilised either the yeast2hybrid (Y2H) assay or affinity pull-down assays with a PTEN fusion protein to identify potential interactors (between 12 and 400 per study), but only a select few (1-4) were taken forward for validation [128-135]. Figure 3 shows the different experimental approaches taken by each of these high-data content investigations. One group expanded the search to identify redox-dependent alteration in PPI through *in vitro* affinity capture with reduced and oxidised PTEN [134]. Of the 97 interactors identified in this study, 4 were further investigated, and of these, the extent of interaction of 3 proteins, peroxiredoxin 1 (Prdx1), thioredoxin (Trx) and annexin A2 (Anxa2), were found to be dependent on the redox status of PTEN [134]. In another study, interactors that had higher affinity for the cancer associated G20E mutant of PTEN were investigated, and 1 specific

G20E interactor, calumenin (CALU) was identified [132]. The systematic approaches to investigating PTEN PPIs of nuclear-localized PTEN, and broader investigations into PPIs of tyrosine phosphatases and their involvement in protein networks, have been recently extensively reviewed [136-139] so have not been described in detail in this section; neither have the genetic techniques of PTEN PPI identification, which have also been reviewed recently [140-142].

Table 2: Summary of the High Data Density Investigations into PTEN's Interactome

Publication Date	Authors (DOI)	Condition	Isolation Method	Identification Method	Validation Method	No. of Potential Interactors	No. of Validated Interactors
2004	Gorbenko et al. [128]	N/A	Y2H	Restriction analysis DNA sequencing	Mating Assay	43	1
2005	Crockett et al. [130] (10.1002/pmic.200401046)	N/A	APD *In silico*	LC-MS/MS	*In vitro* and *in silico* data alignment	*In vitro*: 79 *In silico*: 349	42
2007	Herlevsen et al. [131] (10.1016/j.bbrc.2006.11.067)	Human Bladder Cancer	APD	LC-MS/MS	Reciprocal APD Western Blot	400	1
2008	Ahn et al. [135] (10.1042/BJ20071403)	N/A	APD	LC-MS/MS	Co-IP Western Blot Fluorescence Microscopy RT-PCR	93	1
2010	Gorbenko et al. [129] (10.1007/s11010-009-0312-1)	N/A	Y2H	Restriction analysis DNA sequencing	Co-IP Western Blot SPR	12	1

Table 2: Summary of the High Data Density Investigations into PTEN's Interactome

Publication Date	Authors (DOI)	Condition	Isolation Method	Identification Method	Validation Method	No. of Potential Interactors	No. of Validated Interactors
2011	Gunaratne et al. [132] (10.1074/jbc.M111.221184)	N/A	PAP-SILAC TAP	LC-MS/MS	Co-IP Western Blot Cell Migration Assay	100	4
2011	Maddika et al. [133] (10.1038/ncb2240)	N/A	TAP	LC-MS/MS	Co-IP	34	1
2016	Verrastro et al. [134] (10.1016/j.freeradbiomed.2015.11.004)	Oxidation	APD	LC-MS/MS	Western Blot	97	4

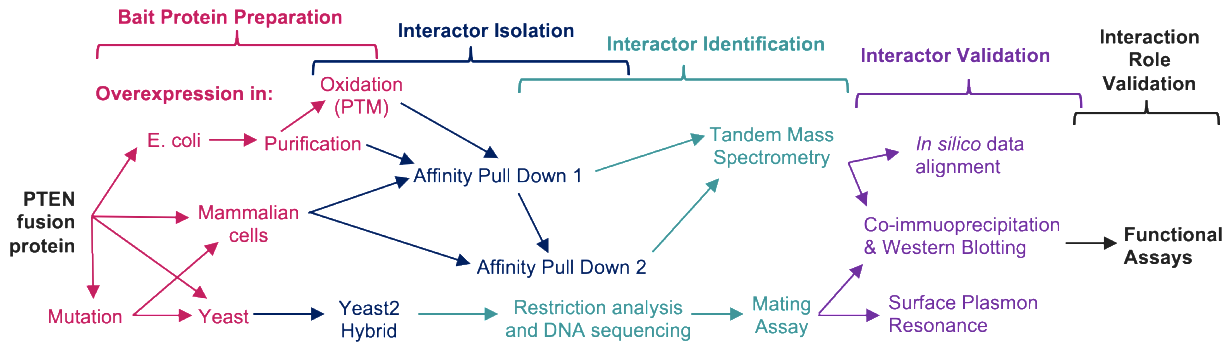

Figure 3. Pathways used in the 8 high data-content investigations into the interactome of PTEN. Once the PTEN fusion protein has been expressed in bait protein preparation and PTMs such as oxidation introduced if required (pink), protein-protein interactors can be isolated (dark blue) and identified (light blue). A small subset of these identified protein-protein interactors can be validated (purple) and for an even smaller subset a functional role may be proposed (black).

3.0 High-Data Content Methods in the Search for Interactors of PTEN

3.1 Affinity Chromatography Assays and Tandem Mass Spectrometry

Out of the 8 investigations into the interactome of PTEN, 6 utilised a tagged or fusion PTEN bait protein and affinity enrichment as the method of PPI isolation (Table 2). Table 3 shows the modified proteins and resins used by each investigation. There are several variations of affinity enrichment that have been used to identify the PTEN PPIs, including affinity pulldown, tandem affinity purification and parallel affinity purification, serving to address different limitations of these methodologies. Affinity pulldown assays use a tagged or fusion protein of interest as bait through immobilisation on a resin support such as sepharose or agarose. The immobilised bait is exposed to potential interactors (the prey proteins), either *in vitro* or through expression of the tagged/fusion bait *in vivo*. The resin is then washed to remove non-specifically bound protein, leaving the binding partners to be eluted along with the bait protein [143]. Binding partners are then

usually identified using tandem mass spectrometry (LC-MS/MS), using quantitative methods, and resin-only controls are required to identify proteins that bind non-specifically to the resin [23, 144].

Tandem Affinity Purification (TAP) follows a similar process to affinity pull down (APD), except the protein of interest is double tagged and the affinity chromatography is performed sequentially, in tandem, using a two-step purification with a resin against each tag [145]. Parallel Affinity Purification (PAP), as with TAP, utilises a double-tagged protein and two resins. However, instead of a two-step purification, two one-step purifications are performed in parallel against each the different tags [146]. Many proteins interact as part of a complex and in some cases non-specific chromatographic techniques, such as ion-exchange and size-exclusion chromatography, have been used to separate these complexes from the initial elution mixture to further fractionate the sample and permit identification of separate complexes [147-149]. Stable Isotope Labelling of Amino Acids in Culture (SILAC) improves the specificity of interactor analysis; the cells must be cultured in media deficient of an essential amino acid and supplemented with normal or isotopically labelled forms of that amino acid to enable the abundance ratio of SILAC peptide pairs from tag only and tag+bait to be compared by mass spectrometry [8, 146, 150]. Interactors that show no change in abundance between the two samples can be assumed to be non-specific.

The general advantages and disadvantages of these different but related techniques have been extensively evaluated [8, 10, 13, 145, 146, 151-155]. All of the affinity chromatography-based techniques are economical and accessible. The whole protein can used as the bait, or regions of the protein can be expressed individually to identify specific domain binding partners. Notable disadvantages include masking of low abundance proteins, as well as false positive and negative results. Table 4 highlights the similarities and differences in the interactions identified between the different techniques. Both affinity pull down and parallel affinity purification can identify weaker and

transient interactors but while tandem affinity purification reduces false positives due to the additional purification step, it also suffers from a higher rate of false negatives for the same reason.

Table 3. Summary of High-Data Density Methods of Protein-Protein Interactor Isolation

Paper	Assay	Fusion Protein	Tag size (amino acids)	Y2H Conditions	Pull Down Conditions	
				Screen System	Resin	Expression Host
Gorbenko et al. [128]	Y2H[A]	Wild type C-terminal PTEN (aa201-403) Phosphatase Dead PTEN (C124S) Mutant PTEN (S370A)	N/A	Duplex-A™	N/A	N/A
Crockett et al. [130]	APD[B]	PTEN-His[!U]	6	N/A	Nickel-Agarose	*E. coli* (not specified)
Herlevsen et al. [131]	APD[B]	PTEN-HA[@C]	31	N/A	Anti-HA Agarose	Mammalian (UMUC-3)
Ahn et al. [135]	APD[B]	PTEN-HA[@U]	31	N/A	Anti-PTEN monoclonal antibody-conjugated agarose	Murine (NIH 3T3)
Gorbenko, et al. [129]	Y2H[A]	C-terminal PTEN (aa201-403)	N/A	Duplex-A™	N/A	N/A
Gunaratne et al. [132]	PAP[C]-SILAC[D] TAP[E]	Wild type: PTEN-FLAP[£U] Mutant: G20E PTEN-FLAP[£U]	243	N/A	Anti-FLAG/Anti-GFP Anti-GFP & Anti-S-protein	Mammalian (LN299 & U87)
Maddika et al. [133]	TAP[E]	PTEN-SFB[$U]	61	N/A	Streptavidin-Sepharose & Anti-S-protein Agarose	Mammalian: (293T)

Table 3. Summary of High-Data Density Methods of Protein-Protein Interactor Isolation

Paper	**Assay**	**Fusion Protein**	**Tag size (amino acids)**	**Y2H Conditions**	**Pull Down Conditions**	
				Screen System	**Resin**	**Expression Host**
Verrastro et al. [134]	APD[B]	PTEN-GST[%N]	211	N/A	Glutathione Agarose	*E. coli* (DH5α)
Assays: A: Yeast2Hybrid (Y2H) B: Affinity Pull Down (APD) C: Parallel affinity purification (PAP) D: Stable Isotope Labelling of Amino Acids in Cell Culture (SILAC) E: Tandem Affinity Purification (TAP)			**Tags:** !: Polyhistidine tag @: Haemagglutinin (HA) tag £: FLAG–Green Fluorescent Protein (GFP)–S-protein (FLAP) tag $: S-protein–FLAG–Streptavidin-binding peptide (SFB) tag %: Glutathione–S–Transferase (GST) tag C: C-terminal tag N: N-terminal tag U: Unknown tag location			

Table 4. Advantages and Disadvantages of Affinity Chromatography Methods

Method	Identifies:				False positives (resin bias)	False negatives (> 1 step purification)
	Direct interactors	Indirect interactors	Weak interactors	Transient interactors		
Affinity Pull Down	✓	✓	✓	✓	✓	↓
Tandem Affinity Purification	✓	✓	↓	↓	↓	↑
Parallel Affinity Purification	✓	✓	✓	✓	↓	↓

Advantages and disadvantages of affinity tags for isolation of proteins and investigation of PPIs have been evaluated previously, from expression and purification conditions to effect on protein properties [156]. A variety of tags have been used for PTEN (Table 3), all of which are suitable for mild purification strategies.

One of the major considerations of the effect of a tag on the protein of interest is correct folding and availability of protein interaction sites. This risk increases with the size and different properties of the tag (Table 3). Whilst the polyhistidine tag, used by Crockett et al. [130] is small and as such less likely to have a negative effect on protein folding or interaction due to size, it suffers from limited specificity, especially over basic proteins, and it can chelate divalent cations. This renders a polyhistidine tag unsuitable for any enzymes requiring divalent cation co-factors, for example magnesium (Mg^{2+}), for activity. Whilst initial studies reported Mg^{2+} as a co-factor for PTEN, on elucidation of its crystal structure Mg^{2+} was not found as part of the PTEN crystal and PTEN was found to be active in Mg^{2+}-free buffers [28, 157], suggesting that it is not a requirement. Divalent cation chelation also occurs with commonly used buffer additives, such as EDTA, and can affect protein-protein interactions that are dependent on calcium (Ca^{2+}) or zinc (Zn^{2+}) cations [13], so care is required in the design of buffer composition.

The GST tag is 243 amino acids in length and was used by Gunaratne et al. and Verrastro et al. [132, 134], whereas Maddika et al. [133] used the 211 amino acid FLAP (FLAG-GFP-S protein) tag. Larger tags have an increased risk of affecting protein properties, such as correct folding, conformation and PPIs. This risk is increased further still with the GST tag due to the potential dimerization of GST [156]. Evaluation of PTEN's catalytic activity with and without GST showed that there was no significant different between the tagged and untagged protein with regards to phosphatase activity towards *p*-nitrophenyl phosphate (PNPP) [158], although this is not the most

sensitive or specific assay for PTEN's phosphatase activity. The phosphatase activity of PTEN-GST was confirmed by another study evaluated here using 3-*O*-methylfluorescein 6-phosphate (OMFP) as a substrate [134]. PTEN has higher affinity for PIP_3 (K_m 30 μM) in comparison to the artificial substrates OMFP (K_m 216 μM) and pNPP (K_m 25,600 μM) [159]. PIP_3 has a molecular weight of 644.2 g/mol, whilst OMFP and pNPP have molecular weights of 426.3 g/mol and 217.1 g/mol, respectively; this decreasing size may result in decreased interference with the tag. However, the conservation of phosphatase activity indicates that protein folding is correct for the substrate binding domains and active site formation.

The location of the tag also needs to be considered, as it may elicit different effects depending on the terminus it is located on. For example, at the catalytic N-terminal domain of PTEN there is a PIP_2 binding domain (amino acids 1-13), whilst the membrane binding C-terminal domain contains the PDZ binding motif (amino acids 401-403). This indicates that a tag on the N-terminus may affect binding to PIP_2 and phosphatase activity, whilst a tag on the C-terminus could affect protein-protein interactions through the PDZ domain and membrane binding. While this has not yet been done for PTEN, one approach to determining impact of the tag on identified PPIs would to trial the tag on both the N and C terminus [160].

The papers reviewed here used two main strategies for PPI isolation. The first involves expression of a PTEN-fusion protein using *Escherichia coli* (*E. coli*) before lysis, purification and subsequent affinity pull down [130, 134]. The second is the expression of the PTEN-fusion protein in mammalian cells, before subsequent lysis and affinity pulldown, negating the requirement of a pre-purification step [131-133, 135]. An advantage of the *E. coli* approach is that the specific phosphatase activity of the PTEN fusion protein can be assessed before affinity pull down. An

additional advantage to this approach in general is that a wide range of proteins can be overexpressed in bacteria at a relatively low cost. In contrast, expressing a PTEN fusion protein in mammalian cells ensures the presence of the appropriate machinery to allow correct folding and PTM of the expressed fusion protein. Protein folding and post-translational modification are a key considerations in the use of an overexpressed protein to search for PPIs, as they are important for protein interactions [161, 162] and as such, key in the identification of physiologically relevant interactors. If a protein is not folded correctly, then regions of proteins that are normally available for PPIs could be hidden and vice versa. Stretches of amino acids, for example hydrophobic regions, that are normally confined to the inner portion of the protein could be exposed leading to an increase in non-specific binding and false positive identification of interactors as well as false negatives. It is worth noting that *E. coli* expressed fusion proteins of *Homo sapiens* PTEN were used in the identification of PTEN's crystal structure as well as catalytic activity, indicating that the lack of mammalian expression machinery with regards to the production of catalytically active, correctly folded PTEN is not prohibitive [28]. There are other advantages to pulling down the PTEN-protein interactor complexes directly from a cellular environment, as described by several groups [131-133, 135], and discussed further in section 3.4.

Once the protein interactors have been isolated by elution of the PTEN-protein complexes from the resin support, identification and quantification of the PPIs can be performed through the use of standard proteomics approaches, including SDS-PAGE and LC-MS/MS. Quantification can be performed by label-free approaches, or isotope labelling methods such as SILAC. In the comparison of a control experiment using unmodified resin (or resin modified with an appropriate control, e.g. an alternative antibody), proteins with very similar abundances can be assumed to be

non-specific interactors, whereas proteins with increased abundance are likely to be specific interactors. LC-MS/MS sequencing of peptides produced by tryptic digestion (or other proteases) is now the most commonly used approach to identification of proteins in complex mixtures, and consequently it also plays a leading role in the identification of PPIs. The advantages and limitations in the use of LC-MS/MS, particularly in the context of affinity purification, have been extensively reviewed [13, 163-165].

3.2 Yeast 2-Hybrid Assay and DNA Sequencing

The basis of the yeast 2-hybrid (Y2H) technique relies on a transcription factor with two functional domains that can be separately expressed as fusion proteins with the protein of interest (bait) and potential interacting proteins (prey) [166]. A common example is use of the Gal4 transcription factor to generate two fusion proteins: a DNA-binding domain Gal4BD+Bait and an activator domain Gal4AD+Prey, which following interaction leads to the transcription of LacZ as a reporter gene, although a variety of different transcription factors and reporting systems can be used [19, 167]. If the bait and the prey are interacting proteins, the two domains of the transcription factor will be in close proximity, activating gene expression of reporter genes [166]. This allows detection of PPIs through permitted growth on restrictive selection media or an observable colour change [18, 166]. As with the affinity purification strategies discussed above, the Y2H assay has advantages and limitations. Firstly, the Y2H relies on the orientation of the interaction between the bait and prey proteins being appropriate to allow the two domains of the transcription factor to achieve a functional interaction [168]. Moreover, whilst Y2H can identify interacting proteins, structural information on this interaction is not produced [168]. One limitation of particular relevance to PTEN, and investigations into cytoplasmic proteins with a role in signal transduction in general,

is that the interactions need to be able to take place within the yeast's nucleus for gene transcription to occur [13, 166]. Other limitations have been cited, as PPIs involving the N-terminal domain of the bait protein are blocked, but this can be overcome using a Y2H system with reversed polarity [13].

The two papers evaluated here that utilised the Y2H system to search for PTEN PPIs originated from the same research group [128, 129]. Both studies used the Duplex-A™ Y2H-system and LexA fused bait proteins. The original paper used 4 constructs, whilst the following paper used only the C-terminal domain of PTEN [128, 129]. In both instances, the PTEN bait proteins were screened by transfected cDNA libraries from Mouse Embryo, HeLa and Colon Cancer cells [128, 129]. Clones identified as positive were subjected to further analysis using a mating assay and identification of the parent cDNA using DNA sequencing. For the full length PTEN, 43 (86%) of the 50 primary positive clone identified from the three libraries (colon cancer, mouse embryo and HeLa) were confirmed by the mating assay [128], although this is decreased to 36 strongly associating clones (72%) when taking account the 7 clones that showed weak attraction, whereas in the assay using the C-terminal region only 27 (13%) of the primary positive clones were confirmed as positive by the mating assay [129].

3.3 Affinity Chromatography vs Yeast2Hybrid: A comparison

A comparison of the affinity chromatography and Y2H approaches to uncovering PPIs has been made in numerous reviews [10, 13, 154], and it is apparent that the nature of the protein and interactions of interest determine which approach is most appropriate. Identification by Y2H is suited to investigations into binary reactions of single, direct protein interactors, whereas affinity purification chromatography has the advantage of being able to isolate protein complexes. Affinity chromatography approaches also work well for proteins participating in signal transduction, where

it might be expected that many protein interactions are transient in nature and often as part of a complex. As mentioned above, there are benefits and limitations to affinity pull down, tandem affinity chromatography and parallel affinity chromatography (Table 4). Notably, with regards to affinity enrichment methods, it is not possible from the initial data to separate primary and secondary interactors, highlighting the importance of validation.

It is widely accepted that PTEN is recruited to a complex of proteins, giving further weight to the benefits of affinity enrichment for its investigation [6, 169]. The benefits of using mammalian or *E. coli* approaches to affinity chromatography have been discussed above although this is influenced by the precise aims of each study. Verrastro et al. sought to identify how the PTEN interactome changes under oxidative stress through treatments with hydrogen peroxide [134]. The PTEN-GST was purified from *E. coli* and either reduced or oxidised before analysis of phosphatase activity, aggregation and binding partners [134]. In this regard, the use of affinity chromatography is more suited than Y2H, and the *in vitro* approach allows the effect of oxidation of the protein itself to be characterized, before the identification of binding partners. While in this study the binding partners identified were from lysates of mammalian cells in normal growth medium, an additional advantage identified in the paper is that lysates from cells subjected to oxidative stress, or indeed other stress conditions, could be used to identify changes in PPIs due to the alterations in the total proteome (e.g. PTMs of interacting proteins) because of the stress condition [134].

3.4 Cellular and Physiological Context

As introduced above, the cell type and physiological status of the cell is likely to have significant impact on PPIs [131-133, 135], as many may occur only during certain cellular events or require specific post-translational modifications. For example, *in vivo* PTEN is phosphorylated by CK2, which

results in inactivation and affects PTEN's recruitment to a complex at the membrane, as discussed in section 2.4 [6]. The dynamic nature of PPIs is illustrated by the changes in the interactome of the scaffold protein 14-3-3β on activation of the insulin-PI3K-AKT pathway [170, 171], and the main interactions between proteins in complexes are non-covalent bonds, which are heavily influenced by the environment in which they are formed [13]. Evaluation of the composition of protein complexes with PTEN by direct IP from mammalian cells should therefore yield data that are more physiologically relevant [13], although it has also been suggested that the use of genetically modified cell lines in high-data content interactome studies may compromise this [171], and it can be difficult to identify the changes to the interacting proteins responsible for the changes in interactome. For all methods, introduction of mutations to the bait, as discussed in section 4.3, or changes to the status of the host cell are also likely to make significant differences.

The (patho)-physiological context may be significantly affected by the cell line being studied, for example whether it is a cancerous or non-cancerous cell line [172] and whether the PTEN is expressed from a plasmid or native genome, as PTEN and other signalling proteins are commonly mutated in cancer cells and signalling pathways may be significantly dysregulated. Seven of the eight papers reviewed here used either mammalian embryonic or cancerous cell lines of various tissue origins for the IP or prey protein source, while only 1 used a non-cancerous cell type (Table 5). Embryonic and cancer cells have many similarities, such as high proliferation rates and a dependence on glycolytic metabolism [173], and therefore might be expected to show some similarities in PTEN AKT signalling status. Interestingly, while most of the cell lines used in the papers reviewed here expressed wild type PTEN, one cell line had a homozygous deletion of PTEN (UMUC-3) and one cell line had a splice mutation (U87) [131, 132, 174]. While there were differences reported in interactions from these studies, the relatively small number of PTEN interactome

studies that have been reported means that it is not possible to draw significant conclusions on interactome differences. Nevertheless, it is likely that they do exist, as a quantitative comparison of the proteome networks of HCT-116, a colon cancer cell line, and 293T, a human embryonic kidney cell line, showed that 54% of the expressed proteome differed between the two cell lines [175], indicating that the protein environment experienced by the bait would be substantially different. The context dependence of PTEN interactors is explored more explicitly in sections 4.3 and 4.4 for mutation and oxidation dependent interactors [132-134].

Table 5. Summary of the Cell Lines used as the Prey Protein Source for Affinity Chromatography

Paper	**Prey Source**		
	Cell Line	**Cell Type**	**PTEN Expression**
Crockett et al. (2005) [130]	Mammalian (SUDHL-1)	Cancer (lymphoma)	Wild type[174]
Herlevsen et al. (2007) [131]	Mammalian (UMUC-3)	Cancer (bladder)	Homozygous deletion (-/-)[174]
Ahn et al. (2008) [135]	Murine (NIH 3T3)	Fibroblast	Wild Type
Gunaratne et al. (2011) [132]	Mammalian (LN299 & U87)	Cancer (glioblastoma)	LN299 – Wild type[174] U87 – Mutation (Splice)[174]
Maddika et al. (2011) [133]	Mammalian: (293T)	Embryonic (kidney)	Wild Type
Verrasto et al. (2016) [134]	Mammalian (HCT-116)	Cancer (colon)	Wild type[174]

4.0 Validated PTEN Interactors

As mentioned previously, both the Y2H and affinity chromatography approaches to investigating PPIs have an inherent risk of false positive identifications. This risk can be mitigated in part

through the use of validation methods to verify that an interaction has taken place, and it is recommended to use more than one method to confirm potential interacting proteins. The most common approach to validation is to confirm the interaction of the two proteins by co-immunoprecipitation confirmed by western blotting, although surface plasmon resonance (SPR) has also been used [18]. Co-immunoprecipitation was utilised by all studies except for the original Y2H investigation by Gorbenko et al. and Crockett et al. [128, 130]. Alternatively, the interaction can be confirmed *in situ*, for example verifying co-localization within the cell using techniques such as fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) [18]. A third approach is to compare *in vitro* and *in silico* data, as used by Gorbenko et al. [128]. Due to the large number of potential protein interactors frequently identified by either affinity chromatography techniques or Y2H, typically only a small number of interactions are actually be validated, and of these a functional role can only be elucidated for a smaller subset still. Figure 4 illustrates the validated PTEN interactors found though high-data content methods and whether a functional role of the interaction has been found. Figure 4 highlights the large number of potential interactors identified through high data-content methods and how this decreases substantially in terms of validated interactors from these studies, decreasing again for those where a functional role of the interaction was proposed. Out of 15 total validated interactors, 11 were PTEN interactors identified previously using more classical biochemical techniques and insights. The high proportion of known interactors identified across these 8 studies provides evidence for the validity of both the Y2H and affinity chromatography methods of PPI identification, but the number of new validated interactors is small. Validated novel interactions included AEBP1, TFG, IQGAP and DDB1 [128, 131, 132, 134]. In order to characterise further the interaction with a specific binding region of PTEN, 2 out of the 8 papers compared interactions of isolated domains of PTEN

with the protein of interest (Figure 4) [128, 133]; identification of where the interactor binds could help to elucidate a function for the interaction. It has been reported that the effect of PPIs on PTEN's phosphatase activity depends on the site of binding; phosphatase activity is enhanced through PPIs with the PDZ motif, whereas a reduction is seen for PPIs with the C2 or phosphatase domain [176, 177]. Functional validation for three interactors, NUDTL16L1, NEDD4-1 and WWP2, was achieved [132, 133], and is discussed further in sections 4.1 and 4.2. The interactions of CALU and WWP2 with PTEN were found to be impacted by PTEN mutation, the interaction of Trx-1, Prdx-1 and Anxa1 were found to be oxidation-dependent. These context-dependent interactors are discussed in section 4.3 and 4.4.

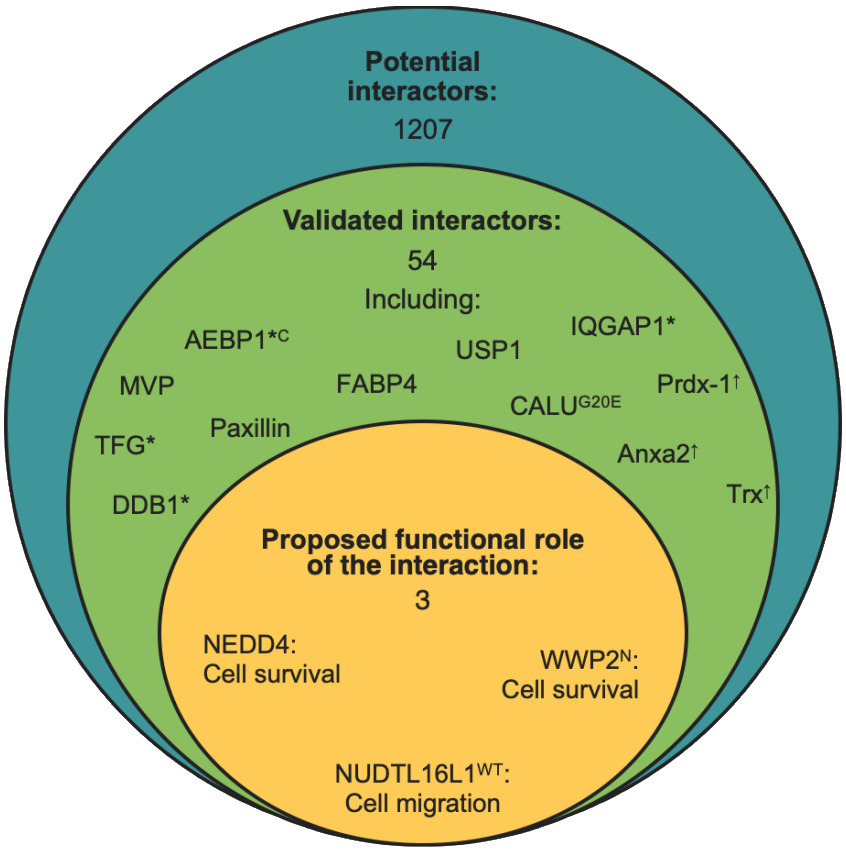

Figure 4. The number of potential PTEN interactors, validated PTEN interactors and interactors with a proposed functional role for the interaction with PTEN. Key: *: a novel PTEN interactor;

↑: an interactor with increased binding to oxidised PTEN; C: an interactor shown to bind to PTEN's C-terminal domain; N: an interactor shown to bind to PTEN's N-terminal domain; G20E: an interactor specific for the G20E PTEN mutant; WT: an interactor specific for wild-type PTEN, not the G20E mutant.

4.1 NUDTL16L1

NUDTL16L1 was identified as having a regulatory role in cell migration through transwell migration assays with cells transfected with either the wild-type or G20E mutant with siRNA against NUDT16L1 or scrambled siRNA [132]. Wild-type cells showed suppressed migration compared to the control, whilst the G20E mutant showed an increase [132]. Cells where NUDT16L1 expression was knocked down using siRNA showed an increase in cell migration compared to the control [132]. When measuring AKT phosphorylation to identify whether this was dependent on activation of the AKT pathway, no difference in levels of phosphorylation was found [132]. It was therefore proposed that regulation of migration by the PTEN-NUDTL16L1 interaction is independent of lipid phosphatase activity [132]. The loss of the PTEN-NUDTL16L1 interaction in the cancer-associated G20E mutant allowed an increase in cell migration [132].

4.2 Ubiquitin ligases: WWP2 and NEDD4-1

WWP2 and NEDD4-1 are HECT-domain-containing E3 ubiquitin ligases with a regulatory role in protein degradation by the 26S proteasome via ubiquitin dependent pathways [133, 178]. The mechanism of action of HECT-family ligases involves transfer of ubiquitin to a substrate lysine residue after donation of ubiquitin from E2 enzymes [178]. The effect of ubiquitination on PTEN is discussed in section 2.4. NEDD4-1 was initially identified as a ubiquitin ligase for PTEN in 2007

[92, 107]. Previous investigations into PTEN nuclear import indicated that mono-ubiquitinylation of PTEN is required and can impart a protective mechanism to preserve the tumour suppressive role of PTEN by avoiding PTEN degradation [92]. A further identification of an interaction between PTEN and NEDD4-1 was achieved in a high-data content study [135], and fluorescence microscopy monitoring of DAPI-staining after transfection with PTEN and/or NEDD4-1 showed that apoptosis was inhibited when NEDD4-1 was co-transfected with PTEN. Immunoblotting showed a reduction in endogenous PTEN on transfection of cells with NEDD4-1, thought to be due to degradation via the UPS system, confirming previous studies reporting NEDD4-1 as a ubiquitin ligase for PTEN [92, 107]. Conversely, cells transfected with PTEN showed a reduction in endogenous NEDD4-1, and mutation of the catalytic cysteine (C124S) of PTEN resulted in loss of down-regulation of NEDD4-and identified that the downregulation of NEDD4-1 is caspase-independent through the PI3K/AKT pathway [135].

The interaction of PTEN and WWP2 was confirmed through co-immunoprecipitation, deletion of either the N- or C-terminal domains of PTEN and repeat co-immunoprecipitation experiments showed that the binding site for WWP2 is the phosphatase domain, more specifically within amino acids 100-187 [133]. The use of ubiquitination assays showed polyubiquitination of PTEN in HeLa cells transfected with wild-type WWP2, but not with a C838A mutant of WWP2 where catalytic activity is abolished [133]. The ubiquitination of PTEN by WWP2 was further demonstrated through *in vitro* assays with PTEN-GST, wild type or mutant WWP2 and an E2 ubiquitin-conjugating enzyme [133], and on siRNA silencing of WWP2 expression ubiquitination of PTEN was decreased [133]. Functionally, the reduction in the levels of PTEN and increase in cellular proliferation on the depletion of WWP2 indicated the PTEN-dependent oncogenic potential of WWP2 [133]. Comparison with the silencing of two further E3 ligases, EDD1 and NEDD4-1, showed that the levels of PTEN

ubiquitination remained unaffected [133]. For this reason, WWP2 was proposed as the lead E3 ligase for PTEN, confirming PTEN as a WWP2 substrate [133]. Recently WWP2 has been shown to be a physiological ubiquitin ligase in mice, acting to promote PTEN degradation *in vivo* [179].

Since the identification of NEDD4-1 as a PTEN ligase, there have been conflicting reports suggesting that both PTEN stability and ubiquitinylation were unaffected in cells deficient in NEDD4-1 [180]. This discrepancy was postulated to be due to the use of overexpression of NEDD4-1 in the earlier papers, whereas in later papers physiological levels of NEDD4-1 were used [180]. Maddika et al also used physiological levels of the E3 ligases NEDD4-1 and WWP2, confirming the WWP2-PTEN interaction but not the NEDD4-1-PTEN interaction [133]. This provided further evidence that initial over-expression of NEDD4-1 could have caused an exaggerated relationship [133, 180]. Direct comparisons of the ubiquitinylation of NEDD4-1 and WWP2 shows that whilst both targeted lysine residues for ubiquitin transfer, WWP2 was more active [181]. At the time of writing, BioGrid [113] includes 14 identifications of NEDD4-1-PTEN and 13 identifications of WWP2-PTEN interactions. WPP2 was not found to have a role in the nuclear import of PTEN, highlighting the multifaceted effect of ubiquitinylation of PTEN within the cell and the divergence of the roles of different E3 ligases [133].

4.3 Mutation Dependent Interactors: CALU and WWP2

PTEN is commonly found to be mutated in cancer [2], and as highlighted in section 3.4, mutations and cellular context, especially those that alter or mimic post-translational modifications, may significantly alter PPIs. Mutations, specifically in the context of endometrial cancer, have been mapped to PTEN and their effect on the 3D structure suggested [182]. It was proposed that PTEN mutations altering the size and properties of the amino acid side could cause changes to the

physical interactions of PTEN [182]; for example, active site mutations (H123Y, R130G, R130Q and R130L) reduce the local environments electrostatic potential, altering specificity of interaction with PIP_3 [182].

Whilst the majority of the high-data content studies searched for wild-type PTEN interactions, the main focus for Gunaratne et al. was to identify whether the interactome of the cancer-associated PTEN mutant G20E was different to wild-type PTEN [132]. Comparison of the wild-type interactome with that of the G20E mutant showed that whilst 2 out of 4 validated interactors were non-specific, only one, CALU, specifically bound G20E PTEN [132]. The G20E specific interactor CALU is a calcium-binding protein involved in the protein sorting and folding functions of the endoplasmic reticulum functions [183].

Although a comparison of wild-type to mutant PTEN was not the main focus for Maddika et al. [133], during validation they sought to identify whether a Y155 mutant of PTEN also interacted with WWP2, as the stability of PTEN has been reported to be affected by tyrosine mutation. Ubiquitinylation by WWP2 of a patient-derived PTEN tyrosine mutant (Y155F) located within the proposed WWP2 binding domain (a.a. 100-182) was analysed, and the Y155F mutant showed increased WWP2 binding, enhanced polyubiquitinylation and a reduced amount of PTEN [133]. These findings led to the proposal that an unknown tyrosine kinase could have a regulatory role in the interactions of WWP2 and PTEN [133].

4.4 Oxidation Dependent Interactors: Trx-1, Prdx-1 and Anxa2

Oxidation of PTEN is widely reported to cause inactivation and altered interactions as discussed in section 2.4. A recent review has evaluated redox regulation of PTEN and the roles of thioredoxin and peroxiredoxin [184]. Previous low-data content investigations into the interactions of thioredoxin

(Trx) and DJ-1 with PTEN identified increased binding on oxidation [118, 185]. Verrastro et al sought to build upon this with a high-data content comparison of the PPIs with oxidised and untreated PTEN [134]. Out of the 3 proteins validated to have increased binding with oxidised PTEN, 2 were the redox proteins thioredoxin-1 (Trx-1) and peroxiredoxin-1 (Prdx-1), previously shown to interact with PTEN. Prdx-1 protects cells from H_2O_2-induced oxidative damage [186]. A previous study reported conflicting data, as decreased binding of Prdx-1 to PTEN was identified during conditions of oxidative stress [123]. This decrease in binding was thought to be due to oxidation of Prdx-1 at C51, and as such is fundamentally different conditions to those used by Verrastro et al., where only the PTEN was oxidised [123, 134]. This could be seen as a limitation of the high-data content search for redox interactors, where only the bait was oxidised, as noted by the authors and described in section 3.1. Verrastro et al. justified the use of this approach as a strength of the investigation, in that this allows a targeted investigation into how the formation of the disulfide bond, a regulatory mechanism in the activity of PTEN, affects PPIs [134]. Further experimentation with oxidised cell lysates, for example, would allow expansion into how oxidation of prey proteins effects PPIs with PTEN. This multi-layered approach is advantageous in providing detail on how oxidation effects PPIs from both the prey and bait perspectives, that broader approaches, such as oxidation of whole lysates and subsequent pull-down of PTEN complexes, wouldn't give. Trx-1 is known to be a physiological reductant of PTEN [7], and an increase in Trx-1 binding to oxidised PTEN was reported to involve Trx-1-PTEN interaction through a C2 domain disulfide bond, inhibiting catalytic activity and increasing tumorigenesis [118]. The third protein identified to have increased binding to oxPTEN was Anxa2, a calcium-dependent phospholipid binding protein [134]. Anxa2 has a diverse role in cellular processes from membrane trafficking to signal transduction and apoptosis [132, 187]. Elevated Anxa2 protein levels are induced by H_2O_2, depletion of which

identified it as a redox regulatory protein with a role in tumorigenesis [187]. Recently, Anxa2 has been shown to interact with PTEN through its C8 residue, upregulating PTEN's negative regulation of the PI3K and inhibiting the Akt pathway[188].

5.0 Summary and perspectives

It can be seen that both the Y2H and affinity chromatography approaches into investigating the interactome of PTEN have been important in the identification and confirmation of a large number of PTEN interactors, although validation by more targeted and specific approaches is needed to increase confidence and demonstrate functional relevance. Using these methods, a G20E mutant-specific interaction has been validated, as well as 3 redox specific interactions. These and other differential interactions may be important in pathophysiological scenarios such as dysregulation of cell proliferation and apoptosis in cancer, or metabolic dysregulation in diabetes and obesity. This emphasises the importance of uncovering the network of PTEN interactors, providing data to corroborate previous studies whilst also generating new information and lines of enquiry. Whilst the interactome of wild-type PTEN has been investigated using various cell lines and fusion tags by different groups, producing data that can be aligned to further increase confidence in the protein hits, the effect of mutations and post-translational modifications on PTEN's interactome is much less well established and represents a significant knowledge gap in the field. In addition, as discussed in section 3.4 the cell type and status, or mutation to the bait, are likely to have significant impact on the interactome, and this is an important and interesting area of study. However, the majority of the small number of studies reported so far have been undertaken with rapidly proliferating cell types under a limited set of conditions, often with overexpression of the bait, so drawing any overall conclusions on the differences between cell types is difficult, and more studies

with different cell types under different growth and stimulation conditions are needed to fully understand the role of the PTEN interactome. Using high data-content approaches and applying them to other mutations and modifications would allow insights into changes to the PTEN interactome that may be brought about by other pathophysiologically important states.

ASSOCIATED CONTENT

Supplementary table 1. Published interactions with PTEN (.xls) lists the 755 published interactions with PTEN curated by BioGRID [113]. For each interaction, the role of the interactor (bait or hit) as well as the organism and experimental evidence is described. The dataset in which the interaction was published is also listed.

Supplementary table 2. Gene Ontology Biological Processes associated with PTEN (.xls) lists the 64 biological processes associated with PTEN, curated by BioGRID [113]. The gene ontology (GO) biological processes listed here refers to both the larger biological processes in which PTEN participates, as well as more specific cellular processes.

Supplementary table 3. Published interactions with PTEN (.xls) lists the 227 published interactions with PTEN curated by IntAct [114]. For each interaction, the role of the interactor (bait or hit) is listed as well as the experimental evidence and the dataset in which the interaction was published.

AUTHOR INFORMATION

Corresponding Author

*Corinne Spickett, School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham, B4 7ET, UK. Tel. +44 (0)121 204 3005. Email: c.m.spickett@aston.ac.uk

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources

This project has received funding from Aston University (UK) and the European Union's Horizon 2020 research and innovation programme under the Marie Sklowdowska-Curie grant agreement number 675132 www.masstrplan.org.

ABBREVIATIONS

AKT, protein kinase B; BRET, bioluminescence resonance energy transfer; CALU, calumenin; CK2, casein kinase 2; DNA, deoxyribonucleic acid; DTT, dithiothreitol; DUP, dual-specificity phosphatase; *E. coli*, *Escherichia coli*; EDTA, ethylenediaminetetraacetic acid; FLAP tag, FLAG-green fluorescent protein-S protein tag; FOXO, forkhead box O; FRET, fluorescence resonance energy transfer; GO, gene ontology; Grdx, glutaredoxin; GSH, glutathione; GSK3, glycogen synthase kinase 3; GST, glutathione-S-transferase; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MDM2, mouse double minute 2 homolog; MMAC-1, mutated in multiple advanced cancers-1; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamycin complex 2; CSNO, S-nitrocysteine; OMFP, 3-*O*-methylfluorescein 6-phosphate; PD-motif, tyrosine/leucine/valine motif; PDK-1, protein kinase 1; PEST sequence. proline/serine/threonine/glutamic acid rich

sequence; PFK2, phosphofructokinase-2; PH domain, plekstrin homology domain; P13K, phosphatidylinositol-3-kinase; PIP_2, phosphatidylinositol-4.5-phosphate; PIP_3, phosphatidylinositol-3,4,5-phosphate; pNPP, *p*-nitrophenyl phosphate; PPI, protein-protein interaction; PTB, phosphotyrosine domain; PTEN, phosphatase and tensin homolog; PTENα, phosphatase and tensin homolog alpha isoform; PTENβ, phosphatase and tensin homolog beta isoform; PTM, post-translational modification; PTP, protein tyrosine phosphatase; rDNA, ribosomal deoxyribonucleic acid; ROS, reactive oxygen species; RTK; receptor tyrosine kinase; SILAC, stable isotope labelling of amino acids in culture; SUMO, small ubiquitin-like modifier; TEP-1, tensin-like phosphatase-1; Trx, thioredoxin; TSC2, tuberous sclerosis complex 2; UPS, ubiquitin protease system; VHR, *Vaccinia* H1-related; Y2H, yeast 2-hybrid

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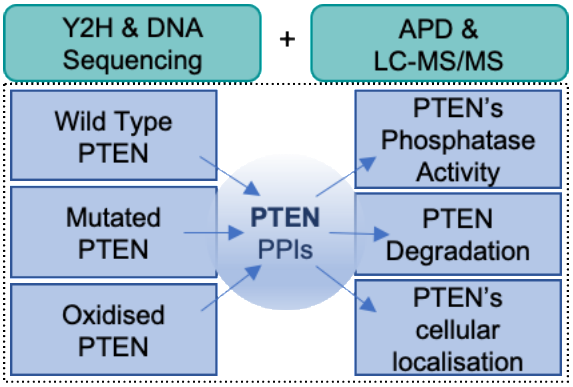

Y2H & DNA Sequencing
+
APD & LC-MS/MS
Wild Type PTEN
Mutated PTEN
Oxidised PTEN
PTEN PPIs
PTEN's Phosphatase Activity
PTEN Degradation
PTEN's cellular localisation

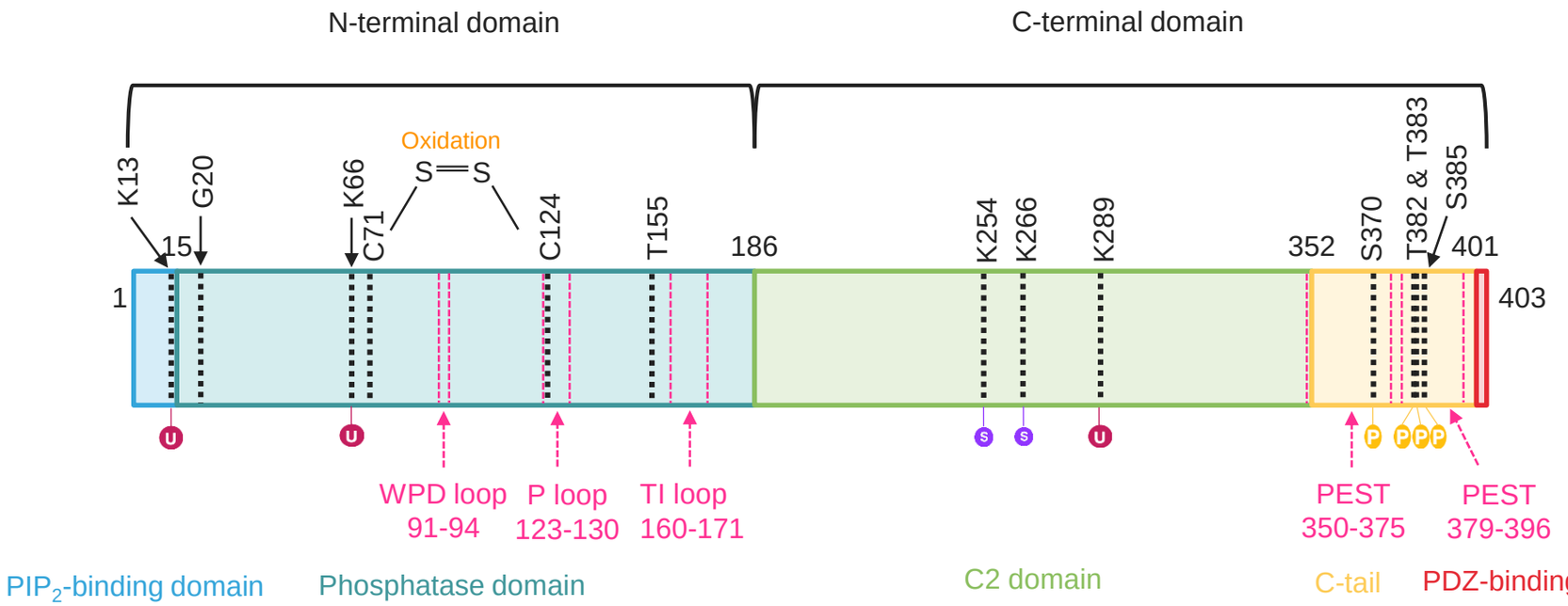
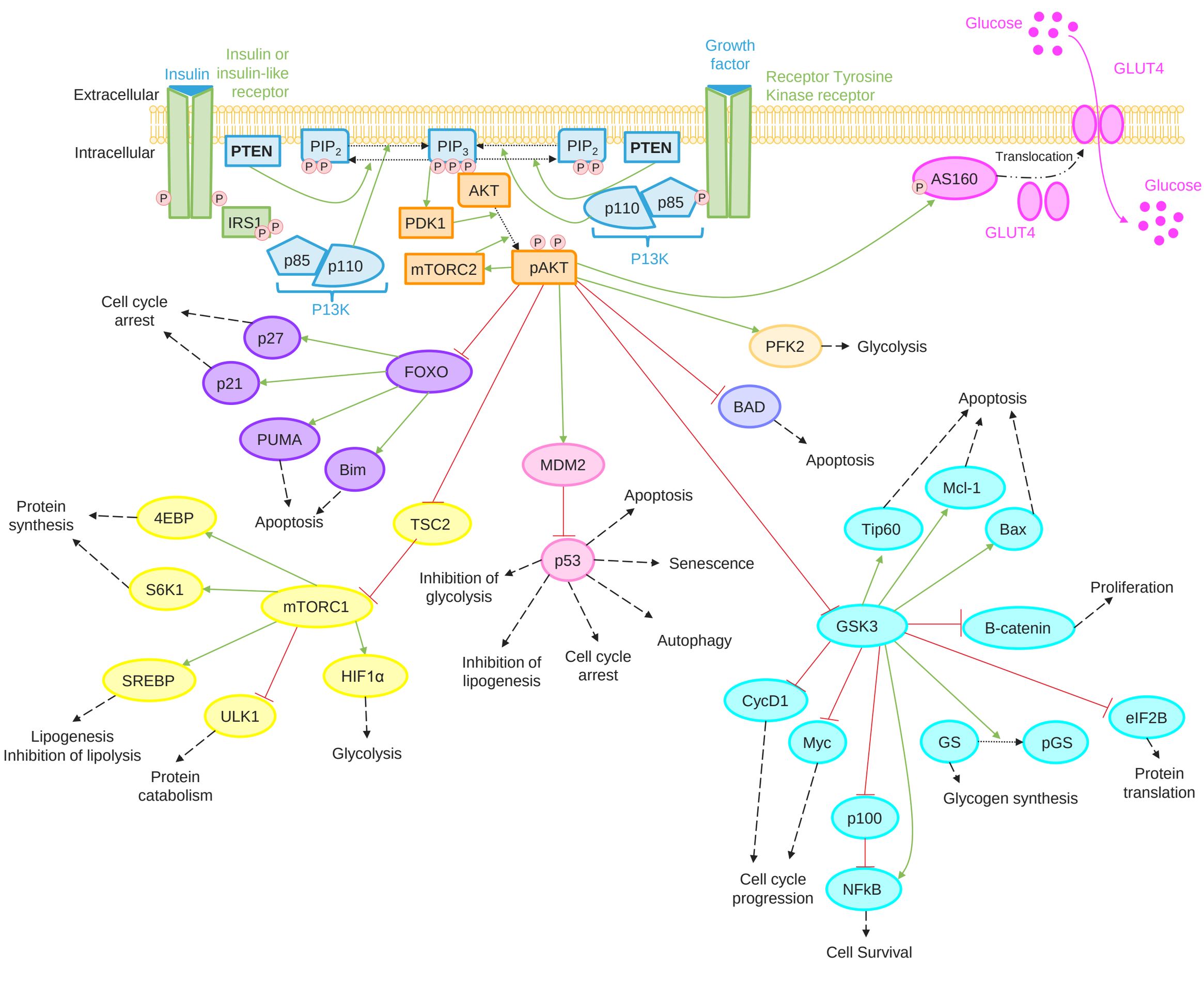


Figure 1: The N- and C- terminal domains of PTEN. The modular structure of PTEN shows N-terminal and C-terminal domains. The N-terminal PIP_2 binding domain (blue) contains K13, which can be modified by ubiquitinylation (U). The phosphatase domain (dark green) contains the WPD loop, P loop and TI loop. Within the phosphatase domain is the resolving C71 and catalytic C124, which on oxidation form a disulfide bond. Two resides, G20 and T55, are mutated in two of the high-data content studies included in this review. The C2 domain (green) contains K254, K266 and K289 which are amino acids modified by ubiquitinylation (U) and SUMOylation (S). The C-terminal tail (yellow) contains two PEST sequences, as well as four residues which can be modified by phosphorylation (P); S370, T382, T383 and S385. The PDZ-binding motif is shown in red.


Glucose
GLUT4
Insulin
Insulin or
insulin-like
receptor
Growth
factor
Receptor Tyrosine
Kinase receptor
Extracellular
Intracellular
PTEN
PIP2
PIP3
PIP2
PTEN
AKT
PDK1
IRS1
p85
p110
P13K
p110
p85
P13K
mTORC2
pAKT
Translocation
AS160
GLUT4
Glucose
Cell cycle
arrest
p27
p21
FOXO
PUMA
Bim
Apoptosis
PFK2
Glycolysis
BAD
Apoptosis
MDM2
Apoptosis
Protein
synthesis
4EBP
TSC2
S6K1
mTORC1
SREBP
ULK1
HIF1α
Lipogenesis
Inhibition of lipolysis
Protein
catabolism
Glycolysis
p53
Senescence
Inhibition of
glycolysis
Autophagy
Inhibition of
lipogenesis
Cell cycle
arrest
Apoptosis
Mcl-1
Tip60
Bax
Proliferation
GSK3
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Myc
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NFkB
Cell cycle
progression
Cell Survival
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pGS
Glycogen synthesis
eIF2B
Protein
translation

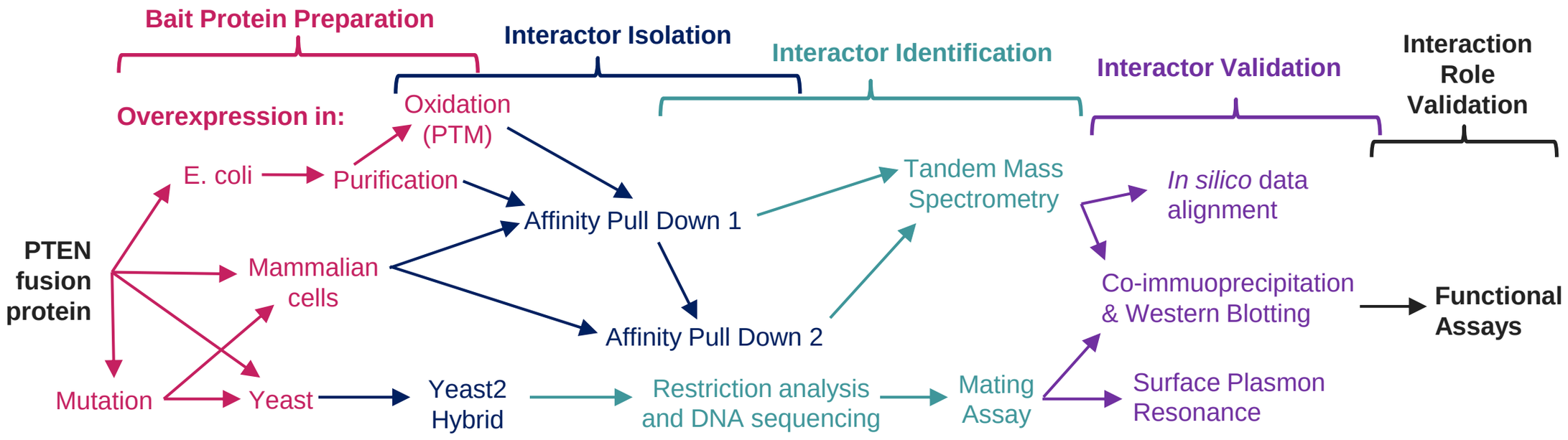

Figure 3. Pathways used in the 8 high data-content investigations into the interactome of PTEN. Once the PTEN fusion protein has been expressed in bait protein preparation and PTMs such as oxidation introduced if required (pink), protein-protein interactors can be isolated (dark blue) and identified (light blue). A small subset of these identified protein-protein interactors can be validated (purple) and for an even smaller subset a functional role may be proposed (black).

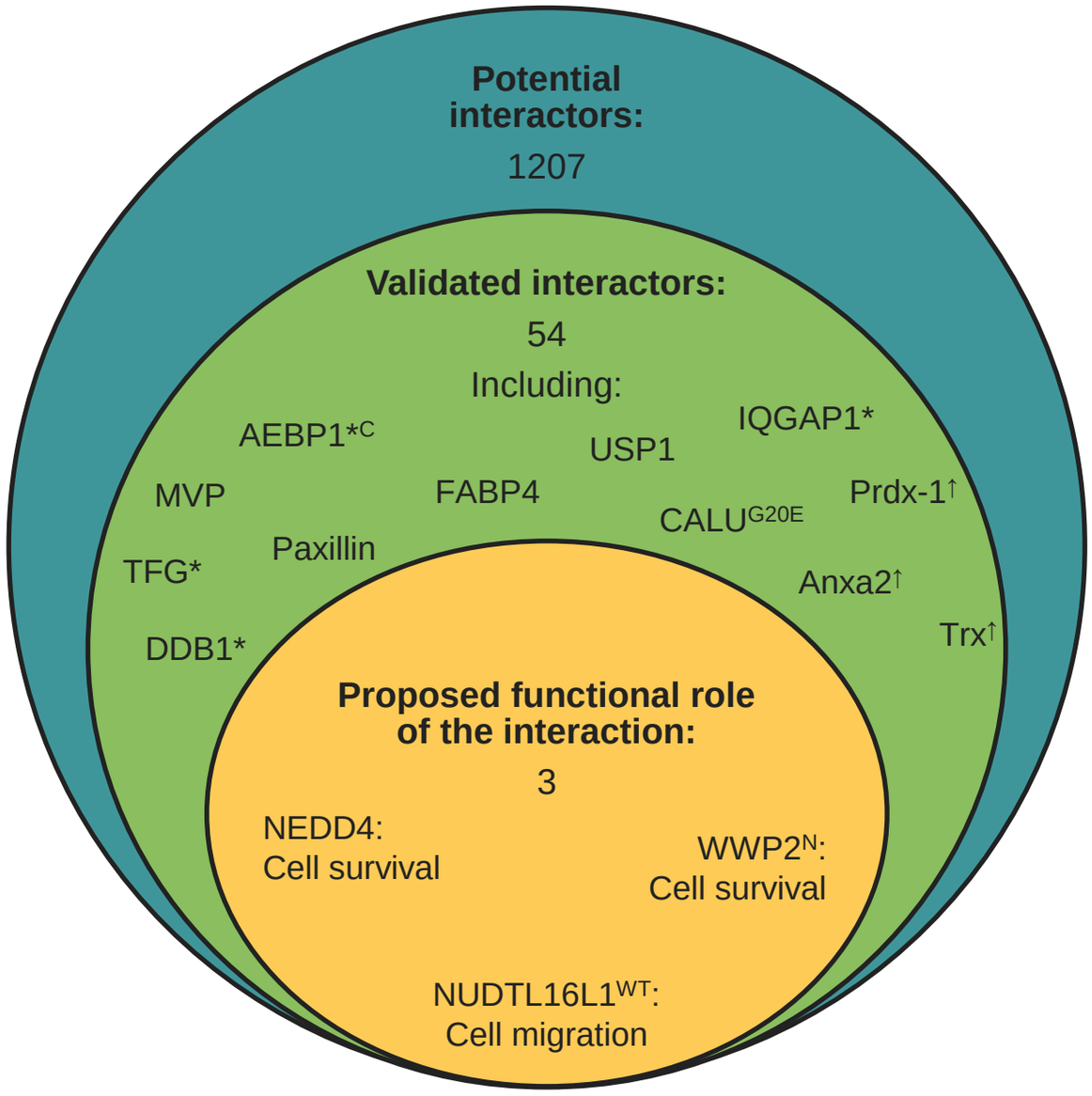

Figure 4: The validated interactors of PTEN and the proposed roles of those interactions.
Key: *: a novel PTEN interactor; ↑: an interactor with increased binding to oxidised PTEN; C: an interactor shown to bind to PTEN's C-terminal domain; N: an interactor shown to bind to PTEN's N-terminal domain; G20E: an interactor specific for the G20E PTEN mutant; WT: an interactor specific for wild-type PTEN, not the G20E mutant.