PIM kinases: From survival factors to regulators of cell motility

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Abstract

PIM kinases are oncogenic serine/threonine kinases, the expression and activities of which are tightly regulated in normal tissues, but upregulated in many types of human malignancies, including both hematological and solid cancers. Since high PIM expression levels have been connected to cancer progression and poor patient survival, PIM kinases have become attractive targets for drug development. Indeed, several selective and potent PIM inhibitors have recently entered clinical trials. Many downstream targets have also been identified, through which PIM kinases promote cell survival, proliferation and metabolism. More recently, PIM kinases have been implicated in regulation of cell motility, which also plays an important role in tumor growth and cancer progression. This review summarizes effects of PIM kinases and their substrates especially on cancer cell migration, invasion and metastatic growth, based on data from cell-based assays, animal experiments and patients.

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1. Introduction – PIM kinases in healthy tissues and cancer

1.1. PIM expression under normal conditions

The PIM family of serine/threonine-specific kinases consists of three members, PIM1, PIM2 and PIM3. As the family acronym indicates, the genes encoding them have all been detected as proviral integration sites for Moloney murine leukemia virus (Cuypers et al., 1984; Breuer et al., 1989; Mikkers et al., 2002), although PIM3 has also been identified as a kinase induced by depolarization (KID1) (Feldman et al., 1998) or by synaptic activity (Konietzko et al., 1999). The three PIM family members form their own phylogenetic niche within the human kinome (Manning et al., 2002), and their catalytically active kinase domains are highly homologous to each other, with over 60% similarity at the amino acid level (The UniProt Consortium, 2017). Based on crystallization or molecular modeling data, the kinase domains of PIM proteins share unique structural features, which make them constitutively active (Qian et al., 2005; Bullock et al., 2009; Ul-Haq et al., 2015). Thus, in contrast to most other kinases, their activity is not dependent on post-translational modifications, but tightly regulated at both transcriptional and translational levels.

The *PIM* genes have partially overlapping mRNA expression patterns in embryonal tissues, especially in cells of the developing immune system, central nervous system and epithelia (Eichmann et al., 2000). In adult tissues, differential expression patterns are also observed, with relatively highest basal levels of *PIM1* in reticulocytes and cells of the upper respiratory tract, *PIM2* in cells of the immune and lymphoid systems, and *PIM3* in bronchial cells and some other epithelial cells (see Supplementary Fig. 1). In hematopoietic cells, expression of both *PIM1* and *PIM2* genes can be transcriptionally induced in cytokine-, hormone- or antigen-dependent fashion, mainly via the JAK/STAT signaling pathways (Dautry et al., 1988; Miura et al., 1994; Buckley et al., 1995; Yip-Schneider et al., 1995; Matikainen et al., 1999; Lehtonen et al., 2002) or NFKB (Li et al., 2001; Zhu et al., 2002a). Accordingly, STAT1, STAT3, STAT4 or STAT5 transcription factors have been shown to directly bind to the *PIM1* promoter and induce expression from there, while NFKB enhances expression of *PIM1* and *PIM2* genes also indirectly via suppression of transcriptional attenuation.

Other upregulators of *PIM* expression include ETS, ERG as well as the Krüppel-like factor 5 (Zhao et al., 2008; Li et al., 2009; Magistroni et al., 2011). Inducible expression is observed also in the brain, where *PIM1* and *PIM3*, but not *PIM2* are upregulated by both physiological and pathological forms of synaptic activity (Feldman et al., 1998; Konietzko et al., 1999)

Production of PIM proteins from their short-lived mRNA transcripts requires cap-dependent translation, which in the case of PIM1 has been shown to be enhanced by binding of the EIF4E initiation factor to both the 5´ and 3´ untranslated regions of *PIM1* mRNA (Hoover et al., 1997; Culjkovic et al., 2006). PIM kinases in turn can promote cap-dependent translation via several mechanisms, including inhibitory phosphorylation of the EIF4E-binding repressor EIF4EBP1, as shown for PIM2 (Fox et al., 2003). Due to the presence of alternative translation initiation sites, several PIM1 and PIM2 isoforms can be produced that differ from each other in both size and stability (Saris et al., 1991; Adam et al., 2015). In general, the half-lives of PIM proteins are relatively short, ranging from 10 minutes to three hours prior to proteosomal degradation, which in the cases of PIM1 and PIM3, but not PIM2 is mediated via ubiquitination and promoted by interactions with the protein phosphatase 2A (Losman et al., 2003; Ma et al., 2007; Adam et al., 2015). In addition, interactions with the heat shock proteins HSP70 and HSP90 negatively and positively regulate PIM1 stability, respectively (Mizuno et al. 2001; Shay et al. 2005). Hypoxic conditions have also been shown to enhance PIM1 stability (Chen et al., 2009). By contrast, oxidative stress can reduce PIM1 expression and anti-oxidants like resveratrol increase it via as yet uncharacterized mechanisms (Lei et al., 2016).

1.2. PIM upregulation in cancer

Since PIM kinases are catalytically active whenever expressed, it is not surprising that their dysregulated expression can have oncogenic consequences. Indeed, overexpressed PIM family members have been implicated in multiple types of human hematological malignancies as well as solid tumors of epithelial origin, such as prostate, pancreatic and gastro-intestinal cancers (Brault et al., 2010; Nawijn et al., 2011). However, there are clear cancer type-dependent differences in their

prevalence (see Supplementary Fig. 2), as exemplified by the relatively high mRNA levels of *PIM1* or *PIM2* observed in lymphoid and myeloid tumors, *PIM1* or *PIM3* in leukemias, *PIM1* in testicular cancer and *PIM3* in prostate cancer. In many types of cancer, *PIM* upregulation has been connected to more advanced and even metastatic cancer stages and to poor patient survival (Table 1). However, as already demonstrated in murine lymphoma models (van Lohuizen et al., 1989; Mikkers et al., 2002), *PIM* overexpression alone is not sufficient to induce tumorigenesis, but requires collaboration with other activated oncogenes, such as *MYC* family members.

Among the hematological malignancies, co-overexpression of *MYC* with *PIM1* or *PIM2* has been observed in the activated B cell-like subtype of DLBCL, the diffuse large B-cell lymphoma (Hoefnagel et al., 2005; Staudt and Dave, 2005), and in the blastoid subtype of mantle cell lymphoma (Zhu et al., 2002b), for both of which the prognosis is worse than for other subtypes. In both murine and human prostate cancer, *PIM1* has been identified as the most consistently upregulated gene associated with the *MYC*-dependent gene expression signature (Ellwood-Yen et al., 2003). Moreover, *PIM1* has been shown to synergize with *MYC* to induce advanced prostate carcinomas in humans and to maintain tumorigenicity of the cancer cells (Wang et al., 2010a; 2012). The most recent addition to the growing list of tumors co-overexpressing *PIM* and *MYC* genes is triple-negative breast cancer, which also has a worse prognosis than other subtypes (Brasó-Maristany et al., 2016; Horiuchi et al., 2016). Thus, co-expression data may be useful for patient stratification.

Even though *PIM* genes are upregulated in several malignancies and behave as oncogenes in many cell and animal models, the correlation between PIM protein overexpression and the clinical outcome of patients varies depending both on the PIM family member and the type of cancer (Table 1). For instance in colorectal cancer, higher PIM1 levels in tumor stroma have been connected to increased survival, but positive PIM3 expression in tumors to decreased survival (Peng et al., 2013; Zhou et al., 2016). In addition, there is heterogeneity within certain cancer types, resulting in contradictory reports on the prognostic role of PIM expression levels. This is especially true for prostate cancer, where positive, negative or no correlations have been reported between clinicopathological parameters and PIM1 levels, which have been observed to be elevated in samples of prostatic

intraepithelial neoplasia or adenocarcinoma as compared to benign prostatic epithelium (Dhanasekaran et al., 2001; Valdman et al., 2004; Cibull et al., 2006; van der Poel et al., 2010). By contrast, upregulation of PIM3 has clearly been connected to high Gleason score and reduced survival of prostate cancer patients (Qu et al., 2016).

As suggested above, the prognostic results may depend on co-overexpressed *MYC* levels, which have been shown to be upregulated together with *PIM1* e.g. during androgen ablation therapy (van der Poel et al., 2010). In addition, they may depend on activities of upstream regulators of *PIM* expression, as exemplified by the observed positive correlation of JAK/STAT activity with prostate cancer stage (Liu et al., 2012). Since PIM kinases have been reported to increase genomic instability in prostate cancer cells (Roh et al., 2003), it is also possible that their overexpression enables cells to acquire additional pro-tumorigenic mutations during cancer progression, but is less advantageous at later stages. Therefore, to make proper conclusions on the prognostic role of PIM kinases in any cancer, it would be important to concurrently determine the expression levels of all PIM family members in different stages of cancer and compare those results to the expression levels of their upstream regulators, collaborating oncoproteins as well as downstream targets.

1.3. PIM inhibitors for research and therapies

The emerging importance of PIM kinases in human tumorigenesis has raised interest in developing small molecule inhibitors against them. Several different classes of PIM inhibitors have been reported (Anizon et al., 2010; Morwick, 2010; Arunesh et al., 2014), but only some of them have shown anticancer activity in cell-based assays or animal models. In addition, only a few of them are effective against all PIM family kinases, even though simultaneous targeting of them all may be necessary for therapeutic purposes. No significant side effects are expected, since mice lacking all three PIM family members are only slightly deficient in their growth responses, but otherwise viable and fertile with a normal life span (Mikkers et al., 2004). However, it is possible that during their development, the knockout mice have found complementary ways to adapt to the loss of PIM expression. Thus, acute PIM inhibition may still have undesired consequences in adult tissues

expressing PIM family members (see Supplementary Fig. 1), especially in the heart, where PIM1 has been shown to have cardioprotective effects (Samse et al., 2016). Indeed, the very first PIM inhibitor in clinical trials, the imidazopyridazine SGI-1776 (Mumenthaler et al., 2009), had to be withdrawn due to cardiotoxicity. This was later demonstrated to be due to off-target effects of the compound, since its more selective derivatives displayed improved safety profiles (Foulks et al., 2014). Yet it is important to study the functions of PIM kinases also in normal tissues to be able to predict possible safety problems with PIM-targeted therapies.

No PIM-selective drugs have FDA approval yet, but several compounds have recently entered into clinical trials to treat patients with relapsed or refractory hematological or solid malignancies (ClinicalTrials.gov, 2017). These compounds include the thiazolidinedione AZD1208 (Keeton et al., 2014), the aminopiperidine LGB321 (Garcia et al., 2014) and the aminocyclohexyl LGH447 (Burger et al., 2015), However, even effective drugs may fail due to development of resistance. Interestingly, PIM kinases have been shown to support chemoresistance in pancreatic, breast and prostate cancer (Xie et al., 2006; Chen et al., 2009; Mumenthaler et al., 2009; Xu et al., 2013; Brasó-Maristany et al., 2016), and radioresistance in head and neck, lung and pancreatic cancers (Peltola et al., 2009; Xu et al., 2011; Kim et al., 2013). This suggests that PIM inhibitors may sensitize tumor cells also to other types of treatments and that combinatorial therapies may therefore be more succesful than single ones. In addition to their clinical value, PIM inhibitors provide useful research tools to study both normal and oncogenic functions of PIM kinases.

1.4. PIM substrates for cell survival, proliferation and metabolism

To understand the physiological functions of any kinase, it is essential to recognize the critical substrates that mediate its effects. In the case of PIM1, which had been shown to phosphorylate serine or threonine residues (Saris et al., 1991), its target sequence was determined already prior to identification of any of its true targets. Based on a library of synthetic peptides, PIM1 was shown to prefer the basophilic sequence K/R-K/R-K/R-X-S/T-X, where X represents an amino acid residue that is neither large and hydrophobic nor basic (Friedmann et al., 1992). By stepwise replacement

of amino acids within the consensus sequence, PIM1 target sites were later defined as K/R-K/R-R-K/R-L-S/T-X, where X represents a small chain amino acid residue (Palaty et al., 1997), or as R-X-R-H-X-S, where X represents any amino acid (Peng et al., 2007). While such a sequence was shown to be targeted also by PIM2 (Peng et al., 2007), similar studies have not been performed with PIM3. However, since several substrates are shared by all PIM family members (Fig. 1), their target sites are expected to be highly homologous to each other.

Multiple approaches have been used to identify PIM kinase substrates, many of which promote or inhibit cell survival or proliferation, and are either positively or negatively regulated by one or more PIM family members (Fig. 1). While the majority of them have initially been identified as PIM1 substrates (Bachmann and Möröy, 2005), it remains to be determined whether most of them are also targeted by PIM2 and/or PIM3. An interaction screen based on the yeast two-hybrid system was used to trap the very first PIM1 substrate, the p100 coactivator (Leverson et al., 1998), which nowadays is known as SND1 (Staphylococcal nuclease and tudor domain containing protein 1). SND1 appeared to act as an adapter to enable PIM1 to find some of its substrates, such as the MYB transcription factor, the activity of which is stimulated by PIM-dependent phosphorylation (Leverson et a., 1998; Winn et al., 2003). SND1 is a multifunctional protein, which has also been implicated as an essential component of the RNA-induced splicing complex mediating RNA interference (Caudy et al., 2003). Interestingly, SND1 is overexpressed in many types of cancer, including androgen-insensitive prostate cancer (Kuruma et al., 2009), suggesting that it may support oncogenic activities of PIM kinases also there.

PIM kinases have also been shown to phosphorylate and thereby enhance the activities of other cellular transcription factors such as NFATC1 (Rainio et al., 2002), RUNX1 (Aho et al., 2006) as well as NOTCH1 (Santio et al., 2016a), all of which are important for production of cytokines that are needed for growth and survival of hematopoietic cells. PIM kinases also promote cytokine-independent survival of cells by several mechanisms, including indirectly upregulated expression of the anti-apoptotic protein BCL2 (Lilly et al., 1999) and direct inactivation of the pro-apoptotic proteins BAD (Yan et al., 2003; Aho et al., 2004; Macdonald et al., 2006), BIM (Xu et al., 2016a) and ASK1

(Gu et al., 2009). These activities explain why the triple knockout mice lacking PIM expression have reduced cytokine responses and remain smaller than their wild-type counterparts (Mikkers et al., 2004), and why the hematopoietic stem cells of these mice are deficient in self-renewal and long-term repopulation (An et al., 2013). Furthermore, they explain why PIM kinases can efficiently co-operate with MYC family transcription factors in tumorigenesis. Even though MYC-overexpressing cells proliferate fast, they are prone to apoptosis. Thus, it is advantageous for them to co-overexpress PIM kinases, which regulate the balance between anti- and pro-apoptotic factors and thereby support cell survival.

PIM-dependent activation of transcription factors can directly or indirectly result in feedback regulation of PIM expression. Among others, positive regulatory loops have been identified between PIM kinases and the NFKB transcription factor (Li et al., 2001; Zhu et al., 2002a; Hammerman et al., 2004; Nihira et al., 2010), the FLT3 receptor tyrosine kinase (Natarajan et al., 2013), as well as NOTCH receptors (Santio et al., 2016a). PIM2 can phosphorylate the IKB kinase COT/MAP3K8 and thereby activate NFKB (Hammerman et al., 2004), whereas RELA, the 65 kD subunit of NFKB, is stabilized by PIM1-dependent phosphorylation (Nihira et al., 2010). Phosphorylation by PIM1 can also enhance the stability of FLT3, especially its cancer-associated constitutively active mutant forms, which are retained in the endoplastic reticulum, resulting in aberrant STAT5 signaling and upregulated PIM expression (Natarajan et al., 2013). Furthermore, all PIM kinases are able to phosphorylate the intracellular domains of NOTCH1 and NOTCH3, while phosphorylated NOTCH1 in turn can enhance PIM expression (Santio et al., 2016a). On the other hand, there are also negative regulatory loops such as that between PIM1 and its substrates SOCS1 and SOCS3, which act as suppressors of cytokine signalling to attenuate JAK/STAT-dependent responses and have been shown to be stabilized by PIM-dependent phosphorylation (Chen et al., 2002; Peltola et al., 2004).

Several key factors that either promote or inhibit cell proliferation are also phosphorylated by PIM kinases (Fig. 1). These include the CHEK1 checkpoint kinase (Yuan et al., 2014), the cell cycle phosphatases CDC25A (Mochizuki et al., 1999) and CDC25C, the CDC25C-associated and TGFβ-activated kinase TAK1/ MAP3K7 (Bachmann et al., 2004), the cyclin-dependent kinase inhibitors

p21/CDKN1A (Zhang et al., 2007; Wang et al., 2010c) and p27/CDKN1B as well as the transcription factors FOXO1 and FOXO3A (Morishita et al., 2008). The observed ability of PIM1 to phosphorylate and thereby stabilize cell surface expression of the multidrug resistance-associated ATP-binding cassette proteins ABCB1 and ABCG2 (Xie et al., 2010, 2008) may in turn contribute to the observed PIM-promoted chemoresistant proliferation of cancer cells.

Highly proliferating normal cells and cancer cells share similar metabolic processes to fuel their growth, such as increased glucose and glutamine uptake as well as aerobic glycolysis and glutaminolysis (Vander Heiden et al., 2009; Lee and Kim, 2016). PIM kinases have been shown to either promote or inhibit the glycolytic phenotype in a context-dependent fashion. In mouse embryonic fibroblasts, PIM3 overexpression was associated with upregulated protein levels for both MYC and PGC-1α (the peroxisome proliferator-activated receptor gamma coactivator 1α), resulting in increased glycolysis and mitochondrial biogenesis (Beharry et al., 2011). Since PIM1 has been shown to phosphorylate histone H3 (H3F3A) at serine 10 at selective MYC-binding sites (Zippo et al., 2007), this may contribute to MYC-dependent activation of genes essential for tumor growth. By contrast, in the highly glycolytic MCF-7 breast cancer cells, PIM1 overexpression was shown to balance cell metabolism to favor oxidative phosphorylation (Santio et al., 2016a). On the other hand, PIM2 was observed to enhance glycolysis in lymphoid cells and in colorectal cancer cells (Fox et al., 2003; Zhang et al., 2015). In lymphoid cells, PIM2 and AKT kinases independently support growth and survival (Fox et al. 2003), but share several substrates such as the pro-apoptotic Bad protein, the EIF4E-binding repressor EIF4EBP1 as well as the MTOR inhibitors PRAS40/AKT1S1 and TSC2, all of which can be phosphorylated and inactivated by either PIM or AKT kinases (Amaravadi and Thompson, 2005; Warfel and Kraft, 2015). While AKT kinases also have other more selective metabolic substrates, those for PIM kinases still remain to be elucidated.

1.5. PIM substrates for cell differentiation and viral transformation

PIM kinases are essential not only for growth and survival, but also differentiation of hematopoietic cells. PIM1 has been shown to support β-selection of pre-T-cells (Schmidt et al., 1998), while all PIM

kinases have been implicated in promoting polarisation of the Th1 subset of activated T helper cells, which are essential in the immune responses against intracellular pathogens (Aho et al., 2005; Tahvanainen et al., 2013). Again, there is a positive feedback loop via upregulation of PIM expression by Th1-polarizing cytokines.

In addition to cellular transcription factors, PIM kinases can regulate activities of viral factors that are essential for reactivation of latent herpesviruses, such as the Epstein-Barr virus (EBV) nuclear antigen EBNA2 (Rainio et al., 2005) and the latency-associated nuclear antigen of the Kaposi´s sarcoma-associated herpesvirus (KSHV) LANA1 (Bajaj et al., 2006; Cheng et al., 2009). Whereas EBV infection induces increased cellular expression of PIM1 and PIM2, these PIM kinases in turn enhance EBNA2 activity via their interactions with SND1 and thereby promote EBV-induced immortalization as well as tumorigenesis (Rainio et al., 2005). In the case of KSHV, PIM1 and PIM3 contribute to viral reactivation via direct phosphorylation of LANA1, which relieves LANA1-dependent transcriptional repression of lytic genes (Bajaj et al., 2006; Cheng et al., 2009). Interestingly, neither EBNA2 nor LANA1 directly bind to DNA, but control gene expression in their host cells by interacting with the NOTCH coactivator CSL (Hayward et al., 2006). Since also NOTCH1 and NOTCH3 are PIM substrates (Santio et al., 2016a), it is possible that PIM-dependent phosphorylation controls the interactions of both cellular and viral factors with CSL.

2. PIM kinases promote cancer cell motility and metastatic growth

2.1. Identification of the pro-migratory role of PIM kinases

The upregulated PIM expression observed in many metastatic solid cancers (Table 1) raises the question of whether PIM kinases have an active role in promoting cell motility. The initial hints towards this direction were obtained from endothelial cells, where RNA interference (RNAi) -based silencing of PIM1 expression decreased cell proliferation and migration (Zippo et al., 2004). This was confirmed in a more recent study, where PIM1 overexpression promoted endothelial cell tube formation and migration (Chen et al., 2016). Both studies recognized the vascular endothelial growth factor (VEGF) as a positive regulator of PIM expression. Also PIM3 silencing reduced migration,

spreading and tube formation in these cells, where PIM3 was shown to localize to the lamellipodia and to colocalize with the focal adhesion kinase (Zhang et al., 2009).

The first piece of evidence to implicate all PIM kinases in regulation of cancer cell motility was obtained from PC-3 prostate cancer cells, where overexpression of any PIM family member increased cell motility in wound healing-based migration assays and in matrigel-based invasion assays (Santio et al., 2010). By contrast, both cell migration and invasion were dramatically decreased, when PIM expression or activity were inhibited by RNAi or by the PIM-selective inhibitor DHPCC-9, respectively (Santio et al., 2010). DHPCC-9 is a cell-permeable pyrrolocarbazole carbaldehyde compound that efficiently targets ATP-binding pockets of all three PIM family members (Akué-Gédu et al., 2009; Letribot et al., 2012). The results with DHPCC-9 were later confirmed with another, structurally unrelated benzo[*cd*]azulene BA-1a, which preferentially targets PIM1 and PIM3 (Kiriazis et al., 2013). Both of these compounds had been validated using a previously described assay (Aho et al., 2004), which measured their efficacy to inhibit PIM-dependent survival of cytokine-deprived myeloid cells without any general cytotoxicity. Similarly to PC-3 cells, DHPCC-9 and BA-1a also inhibited motility of head and neck squamocellular carcinoma cells (Santio et al., 2010; Kiriazis et al., 2013). Thereafter, multiple studies in different cell types have demonstrated that PIM overexpression can promote cell motility, while PIM silencing or inhibition can reduce it (Table 2).

2.2. Upstream regulators of PIM-promoted cell motility

Any factors regulating PIM expression or stability may indirectly affect cell migration and/or invasion. For instance, TNF (tumor necrosis factor) has been shown to stabilize *PIM3* mRNA and thereby promote endothelial cell sprouting and angiogenesis (Yang et al., 2011). In addition, there are multiple microRNAs that have recently been shown to downregulate both *PIM* mRNA levels and cell motility. These include *PIM1*-targeting microRNAs *miR-1*, *miR-214* and *miR-486-5p* in lung cancer (Pang et al., 2014; Amatya et al., 2016;), *miR-33b* in multiple myeloma (Tian et al., 2012), *miR-101-3p* in salivary gland carcinoma (Liu et al., 2015), *miR-124-3p* in astrocytoma (Deng et al., 2016), *miR-206* in mesenchymal stem cells (Zhang et al., 2016), *miR-328* in pulmonary arterial smooth

muscle cells (Qian et al., 2016) and *miR-542-3p* in melanoma (Rang et al., 2016). Similarly, the *PIM3*-targeting microRNA *miR-377* decreases migration of pancreatic cancer cells (Chang et al., 2016). While these results suggest that decreased *PIM* mRNA levels inhibit cancer cell motility as well as angiogenesis, it is necessary to keep in mind that these microRNAs may also target other pro-migratory factors than the PIM family members.

2.3. PIM substrates for cell motility

Several PIM kinase substrates have been implicated in the regulation of cell motility (Fig. 2a). By phosphorylating these proteins or by indirectly regulating other factors, PIM kinases can promote not only cancer cell motility, but also hematopoietic cell migration and angiogenesis-associated endothelial cell behaviour, as described below.

The first direct substrate connected to PIM-stimulated cell motility was CXCR4, the C-X-C motif chemokine receptor 4 (Grundler et al., 2009), which is essential for homing of hematopoietic stem cells (Suarez-Alvarez et al., 2012). When bone marrow cells were transplanted into mice, PIM1 silencing led to reduced blood cell and platelet counts, suggesting a role for PIM1 in the regulation of bone marrow reconstitution (Grundler et al., 2009). Furthermore, PIM1, but not PIM2 was shown to phosphorylate CXCR4 on Ser339, leading to increased cell surface expression of the receptor and enhanced migration of cells towards the CXCR4 ligand CXCL12. This was in line with the observed inability of PIM2 to substitute for PIM1 in hematopoietic cell homing and migration assays (Grundler et al., 2009).

The CXCR4/CXCL12 pathway can support migration of both normal and malignant hematopoietic cells. In AML (acute myelogenous leukemia), DLBCL (diffuse large B-cell lymphoma) and CLL (chronic lymphocytic leukemia) cells, PIM1 expression levels were shown to correlate with hyperphosphorylation of CXCR4, while PIM1 downregulation or inhibition in these cells decreased cell surface expression of CXCR4 and thereby also cell migration and homing (Grundler et al., 2009; Brault et al., 2012; Decker et al., 2014). The CXCR4/CXCL12 pathway is known to drive migration and invasion of also adherent cancer cells (Vaday et al., 2004; Hart et al., 2005) and help them to

form site-specific metastases (Ben-Baruch, 2009). Accordingly, PIM silencing or inhibition were shown to inhibit PC-3 prostate cancer cell invasion towards a medium containing CXCL12, while both PIM1 and PIM3 phosphorylated CXCR4 *in vitro* and in PC-3 cells (Santio et al., 2010, 2015).

Since then, several other PIM substrates have been shown to contribute to the pro-migratory effects of PIM kinases, including transcription factors NFATC1, FOXP3 and NOTCH1 as well as GSK3B kinase (see below). Although NFATC1 was originally identified as a nuclear factor of activated T cells regulating immune responses, it has more recently been implicated also in cancer progression and even in invasive migration of epithelial cells (Mancini and Toker, 2009). Furthermore, NFATC1 is highly expressed in PC-3 and other prostate cancer cells, but not in normal prostate tissues (Kawahara et al., 2015; Manda et al., 2016). Similarly to PIM kinases, overexpressed NFATC1 promotes PC-3 cell motility (Santio et al., 2010). However, the pro-migratory effects of NFATC1 are completely abolished by the PIM inhibitor DHPCC-9, suggesting that PIM-dependent phosphorylation is required for full activity of either NFATC1 itself or some other downstream effector(s) shared by both PIM and NFATC1.

One such effector may be the prostaglandin-endoperoxide synthase 2 (PTGS2, also known as cyclooxygenase-2, COX2), which is an NFATC1 target gene (Hernandez et al., 2001), and required for its ability to promote invasive migration of breast cancer cells (Mancini and Toker, 2009). PTGS2 is a pro-inflammatory and pro-angiogenic factor, which catalyzes the production of prostaglandins from arachidonic acid, but which has also been implicated e.g. in prostate cancer (Hussain et al., 2003). PTGS2 expression is upregulated in PIM1-overexpressing PC-3 cells, and downregulated by inhibition of PIM expression or activity (Santio et al., 2016b). PTGS2 activity in turn is required for PIM-dependent cell migration, which is selectively reduced by the PTGS2 inhibitor Celecoxib, but not by the PTGS1 inhibitor Indomethacin.

In gastric cancer cells, both GSK3B and FOXP3 have been reported to inhibit PTGS2 expression as well as cell migration (Thiel et al., 2006; Hao et al., 2014). Interestingly, both of these proteins can be phosphorylated and thereby inactivated by PIM kinases. The FOXP3 (forkhead box P3)

transcription factor is a biomarker for regulatory T cells, where it has been shown to support PIM2 expression (Basu et al., 2008). However, it can also act as a tumor suppressor, and its X-linked gene has often been found to be inactivated in breast and prostate cancer (Wang et al., 2010b). PIM2 has been shown to negatively regulate the immunosuppressive functions of regulatory T cells by phosphorylating FOXP3 at multiple N-terminal residues (Deng et al., 2015), while two C-terminal close-by sites, Ser418 and Ser422 have been identified as PIM1 target sites (Li et al., 2014; Santio et al., 2016b). While the differential selectivity of PIM family members towards distinct FOXP3 sites remains to be confirmed, PIM1-dependent phosphorylation regulates the ability of FOXP3 to inhibit migration of PC-3 cells (Santio et al., 2016b). Mutation of the Ser422 residue to a non-phosphorylatable alanine increases the inhibitory functions of FOXP3, while a similar mutation of Ser418 completely inactivates it. However, the Ser422 residue is not conserved between human and mouse, which may explain the stronger anti-migratory effects observed by overexpression of the mouse protein as compared to its human counterpart. By contrast, phosphorylation of the conserved Ser418 residue appears to be required for proper DNA-binding and tumor-suppressive functions of both human and mouse proteins (Nie et al., 2013; Santio et al., 2016b).

The GSK3B kinase in turn is phosphorylated by PIM1 on the negative regulatory site Ser9 (Santio et al., 2016b), which is targeted also by other kinases, including PKB/AKT (Cross et al., 1995) and PKA (Fang et al., 2000). Mutation of this residue to alanine strongly enhanced the inhibitory effects of GSK3B on migration of PC-3 cells (Santio et al., 2016b). Furthermore, phosphorylation of GSK3B, but not FOXP3, relieved repression of PTGS2 expression in these cells.

Deregulated NOTCH signaling has been observed in both hematological and solid malignancies, where it is associated with increased migration, invasion and metastatic growth in a context-dependent fashion (Andersson and Lendahl, 2014; Carvalho et al., 2014). The NOTCH receptors are phosphoproteins, but the roles of post-translational modifications in modulating their activities have not yet been fully explored. However, all three PIM family members have recently been shown to phosphorylate NOTCH1 (Santio et al., 2016a). The PIM target site identified from the mouse protein is Ser2152, which corresponds to Ser2162 in the human protein. This site is next to the

nuclear localization domain, which is needed for translocation of the NOTCH1 intracellular domain (N1ICD) into the nucleus after activation and cleavage of the full-length transmembrane receptor. Accordingly, PIM-dependent phosphorylation was shown to promote the nuclear translocation of N1ICD as well as its transactivation activity towards its targets genes, including *PIM* genes (Santio et al., 2016a). Moreover, activation of endogenous NOTCH signaling in PC-3 cells by its natural ligand Jagged1 enhanced cell migration. Similar effects were induced by overexpression of wild-type NOTCH1, but not by the corresponding phosphorylation-deficient mutant. By contrast, inhibition of the cleavage of endogenous NOTCH1 decreased cell motility to the same extent as PIM inhibition, indicating that activities of both PIM kinases and NOTCH1 are needed to efficiently support prostate cancer cell migration.

PIM1 has also been shown to positively regulate invasiveness of salivary gland carcinoma cells, where PIM1 silencing resulted in upregulated RUNX3 expression (Zhu et al., 2014; Liu et al., 2015). In prostate cancer cells, RUNX3 upregulation can in turn inhibit cell migration and invasion, angiogenesis and the formation of metastases (Chen et al., 2014). Interestingly, PIM-dependent phosphorylation of RUNX family members has been shown to have opposite effects on the transcriptional activities of RUNX1 and RUNX3 (Aho et al., 2006; Kim et al., 2008). RUNX1 activity is increased via C-terminal phosphorylation, whereas N-terminal phosphorylation of RUNX3 results in its transport out of the nucleus.

While PIM kinases have been implicated in the regulation of endothelial cell migration and angiogenesis (Zippo et al., 2004; Zhang et al., 2009), only one PIM substrate so far has been directly connected to this. In vascular endothelial cells, PIM1 was shown to promote tube formation and cell migration via phosphorylation-dependent activation of NOS3, the endothelial nitric oxide synthase (Chen et al., 2016).

2.4. PIM substrates for cell adhesion and epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a crucial phenomenon during embryogenesis, but also plays an important role in cancer cell motility (Moustakas and Heldin, 2007). During EMT, cell

adhesion is changed and several well-known changes can be detected in cell cytoskeleton such as decreased levels of the cell-cell adhesion protein E-cadherin (CDH1) and increased levels of N-cadherin (CDH2) and the intermediate filament vimentin (Moustakas and Heldin, 2007; Jeanes et al., 2008).

In many malignant tumors, EMT is induced via interactions between the hepatocyte growth factor (HGF) produced by stromal cells and its receptor MET expressed by cancer cells (Benvenuti and Comoglio, 2007; Spina et al., 2015). Via phosphorylation of the eukaryotic translation initiation factor EIF4B, PIM1 was shown to promote activation of the HGF/MET pathway and thereby also EMT and cell migration in several types of cancer cells (Cen et al., 2014). This correlated well with the observed co-overexpression of PIM1 and MET in samples derived from prostate cancer patients. In breast cancer cells, induction of EMT was shown to result from constitutive activation of STAT3 via a PIM2-driven positive feedback loop (Uddin et al., 2015). By contrast, PIM2 silencing decreased cell motility and downregulated expression of the EMT markers N-cadherin and vimentin. However, except for EIF4B, other relevant PIM substrates regulating EMT are yet to be identified.

The role of PIM kinases in cell adhesion has been investigated in both normal endothelial cells and in cancer cells. Mouse aortic endothelial cells derived from PIM1 knockout mice showed decreased cell migration as well as increased cell-cell and cell-substratum adhesion as compared to cells from control mice (Walpen et al., 2012). This correlated with increased expression of adhesion-associated genes together with stronger clustering of vinculin and β-catenin, which are important mediators of cell adhesion. Vinculin is an actin-binding protein present in focal adhesions, while β-catenin is a binding partner for E-cadherin (Jeanes et al., 2008; Walpen et al., 2012). In prostate cancer cells, treatment with the pan-PIM inhibitor DHPCC-9 decreased integrin-dependent adhesion and spreading of cells on collagen and fibronectin matrices (Santio et al., 2016b). However, cell surface expression or activity of integrins remained unaffected, suggesting involvement of other, as yet uncharacterized mechanisms.

2.5. Animal models to study PIM-mediated motility and angiogenesis *in vivo*

Multiple *in vivo* experiments also support the pro-migratory role of PIM kinases in cancer (Fig. 2b). The first study connecting PIM kinases to the formation of metastases was performed using a mouse model for sarcoma, where 3-methylcholanthrene was injected into mouse muscles to create invasive tumors (Narlik-Grassow et al., 2012). In knockout mice lacking two or three PIM family members, sarcomas developed more slowly and were much less invasive than in control mice, which correlated with decreased levels of matrix metalloprotease 9 and reduced phosphorylation of GSK3B (Narlik-Grassow et al., 2012), which is a PIM substrate (Santio et al., 2016b).

In both subcutaneous and orthotopic nude mouse xenograft models for human pancreatic cancer, overexpression of PIM3 was shown to enhance tumor growth and neovascularization via upregulation of intratumoral expression of angiogenic factors, such as VEGF and HGF (Wang et al., 2013; Liu et al., 2014). However, no metastases were observed in either case. By contrast, when PC-3 prostate cancer cells overexpressing either PIM1 or PIM3 were orthotopically inoculated into prostates of nude mice, cells from the xenograft tumors migrated to the lungs, where they formed metastatic colonies (Santio et al., 2015). The more slowly growing tumors formed by the parental cells were not able to move further than to the nearest prostate-draining lymph nodes, as also previously observed (Tuomela et al., 2008). In addition, PIM overexpression supported both angiogenesis and lymphangiogenesis, which may have increased the invasive potential of cancer cells (Santio et al., 2015). Furthermore, both PIM1 and PIM3 enhanced phosphorylation and cell surface expression of CXCR4, which may have facilitated the movement of cells towards CXCL12-expressing tissues such as the lungs to form metastases there. In addition, PIM overexpression correlated with increased nuclear levels of activated NOTCH1 (Santio et al., 2016a), suggesting that this PIM substrate is essential for the pro-migratory effects of PIM kinases not only in cultured cells, but also under *in vivo* conditions. Similarly to the orthotopic prostate cancer model, PIM1 has also been connected to the formation of lung metastases in mouse models for human hepatocellular carcinoma and melanoma, where *in vivo* migration, invasion and metastatic growth of cancer cells have been inhibited by PIM1 silencing (Leung et al., 2015; Rang et al., 2016).

In addition to these cancer models, there are *in vivo* data supporting the ability of PIM kinases to promote angiogenesis also under normal physiological conditions (Chen et al., 2016). When aortic rings from healthy and diabetic mice were compared in *ex vivo* microvessel sprouting assays, the expression levels for endogenously expressed PIM1 and its substrate NOS3 were clearly lower in the diseased animals, correlating with significantly diminished cell sprouting. By contrast, adenovirus-mediated overexpression PIM1 was able to increase sprouting in both types of mice.

So far only few PIM-selective inhibitors have been shown to decrease the metastatic potential of cancer cells *in vivo*. In the orthotopic mouse model for human prostate cancer, daily treatment with the pan-PIM inhibitor DHPCC-9 drastically decreased not only tumor growth, but also the metastatic potential of PIM-expressing xenografts without any overall signs of toxicity (Santio et al., 2015). The DHPCC-9 treatment also suppressed angiogenesis and lymphangiogenesis, and reduced activities of the PIM substrates CXCR4 and NOTCH1, supporting their critical roles in mediating the pro-migratory effects of PIM kinases during prostate cancer progression *in vivo* (Santio et al., 2015, 2016a). This conclusion was further supported by the observation that combined inhibition of endogenous PIM and NOTCH activities suppressed growth of prostate cancer cells on top of chick embryo chorioallantoic membranes (CAM) more efficiently than individual treatments (Santio et al., 2016a). The chick embryos are naturally immunodeficient hosts for xenografts and provide a thin three-layer structure with uniquely supportive vascularized environment to nurture xenograft growth (Kunzi-Rapp et al., 2001). Thus, both the orthotopic prostate cancer model and the CAM model have proven to be useful methods to measure the *in vivo* efficacy of PIM kinase inhibitors.

Another pan-PIM inhibitor, the oxindolo-furanyl compound CX-6258 was identified and tested in leukemia and prostate cancer models in mice (Haddach et al., 2011). Later on, it was shown to suppress proliferation and invasive potential of also human patient-derived metastatic prostate cancer xenografts (Rebello et al., 2016). However, alone it was much less effective than in combination with the RNA polymerase I inhibitor CX-5461. While it remains to be determined whether other PIM inhibitors also have anti-metastatic effects, it was promising to notice that compounds such as AZD1208 and LGB321 could not only prevent tumor formation, but also reduce

the sizes of pre-existing breast or prostate xenograft tumors and sensitize them to chemo- or radiotherapy (Kirschner et al., 2015; Brasó-Maristany et al., 2016; Horiuchi et al., 2016). However, since a phase I clinical trial with AZD1208 failed to demonstrate any benefit for patients with advanced solid tumors or malignant lymphoma (ClinicalTrials.gov, 2017), this suggests that combinatorial approaches are needed both to improve drug efficacy and to avoid resistance that is easily acquired via intrinsic mechanisms. As demonstrated by a recent study, where suppression of the p38 kinase pathway was shown to synergize with PIM inhibition by AZD1208 (Brunen et al., 2016), screens based on synthetic lethality provide a powerful strategy for identification of novel therapeutic vulnerabilities and for finding effective drug combinations without overly severe side-effects (Brunen and Bernards, 2017).

3. Concluding remarks

Recent data indicate that PIM kinases regulate not only cell survival, but also several other cellular functions, such as cell motility. By enhancing tumor growth, angiogenesis and motility, PIM kinases can contribute to fatal formation of metastases. Therefore, it is important to continue the ongoing development of PIM-targeted therapies against both hematological and solid malignancies. To be successful, it is crucial to identify those patients that would best benefit from PIM-targeted therapy. Stratification of patients based on co-overexpressed collaborators of PIM kinases, such as MYC, may be helpful there. It will also be essential to identify such direct or indirect PIM substrates that could be used as biomarkers for PIM activity. Furthermore, since PIM kinases contribute to chemo- and radioresistance of tumors, it will be necessary to test PIM inhibitors in combination with other therapies, taking also into account possible PIM-induced vulnerabilities within the target tissues.

While several substrates have already been shown to mediate the effects of PIM kinases on cell growth, metabolism and survival, most of those relevant for regulation of cell motility and adhesion are yet to be identified. Since the majority of studies so far have only addressed PIM1 and its targets, it will be essential to determine which of the substrates are truly shared by all PIM kinases, and which are more selectively targeted. In this respect, there is already evidence to indicate that despite the

high homology between the catalytically active kinase domains of PIM family members, PIM2 behaves differentially from the two others in terms of both substrate selectivity and druggability. This in turn may help to avoid severe side-effects, especially in therapies against the many types of solid cancers, where PIM1 or PIM3 have been more strongly implicated. In any case, accumulating data on the upstream regulators and downstream effectors of PIM kinases are expected to nurture novel approaches towards improved therapies, even against therapeutically highly challenging cases such as castration-resistant prostate cancer and triple-negative breast cancer. Furthermore, the PIM-targeted chemical inhibitors as well as microRNAs provide prominent research tools to increase our understanding of the PIM-dependent signaling pathways regulating the fate and physiological functions of both normal and cancer cells.

Conflict of interest

Authors declare no conflict of interest.

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Figure and Table legends

Fig. 1. PIM kinase substrates in cell survival

PIM family kinases support cell survival and tumor growth by positively or negatively regulating a large variety of substrates. While it is likely that most of these substrates can be targeted by all three PIM family members in a context-dependent fashion, it should be noted that in most reported cases, phosphorylation studies have been carried out with only one family member, usually PIM1.

Fig. 2. PIM kinase substrates in cell motility

a, So far only a few PIM substrates have been identified as mediators of the pro-migratory effects of PIM kinases. **b**, Several mouse models have been used to study the role of PIM kinases in the regulation of cancer cell motility *in vivo.* *PIM inhibitor CX-6258 was used in combination with RNA polymerase I inhibitor CX-5461. See text for further details and references.

Table 1. Association of PIM expression levels with clinical outcome in cancer patients

PIM mRNA and/or protein levels in solid cancers were determined from patient samples. In all listed cases, PIM1 or PIM3 levels were upregulated in cancerous tissues as compared to healthy tissues. In most cases, PIM upregulation correlated positively with tumor size/stage, metastases and/or poor patient survival (+). However, in some cases, correlation was not detected or determined (-).

Table 2. PIM kinases support cell migration in various cell types

PIM kinases have been indicated as regulators of cell migration and/or invasion in several cell-based studies, where expression levels of indicated PIM family members were modified either *up* or *down* (x), and/or PIM activity was decreased by *inhibition* with indicated chemical compounds. In all listed cases, PIM upregulation increased cell motility and PIM downregulation or inhibition decreased it.

Supplementary Data

Supplementary Figures 1 and 2 along with their figure legends can be downloaded as a separate PDF file (SupplementaryData.pdf).

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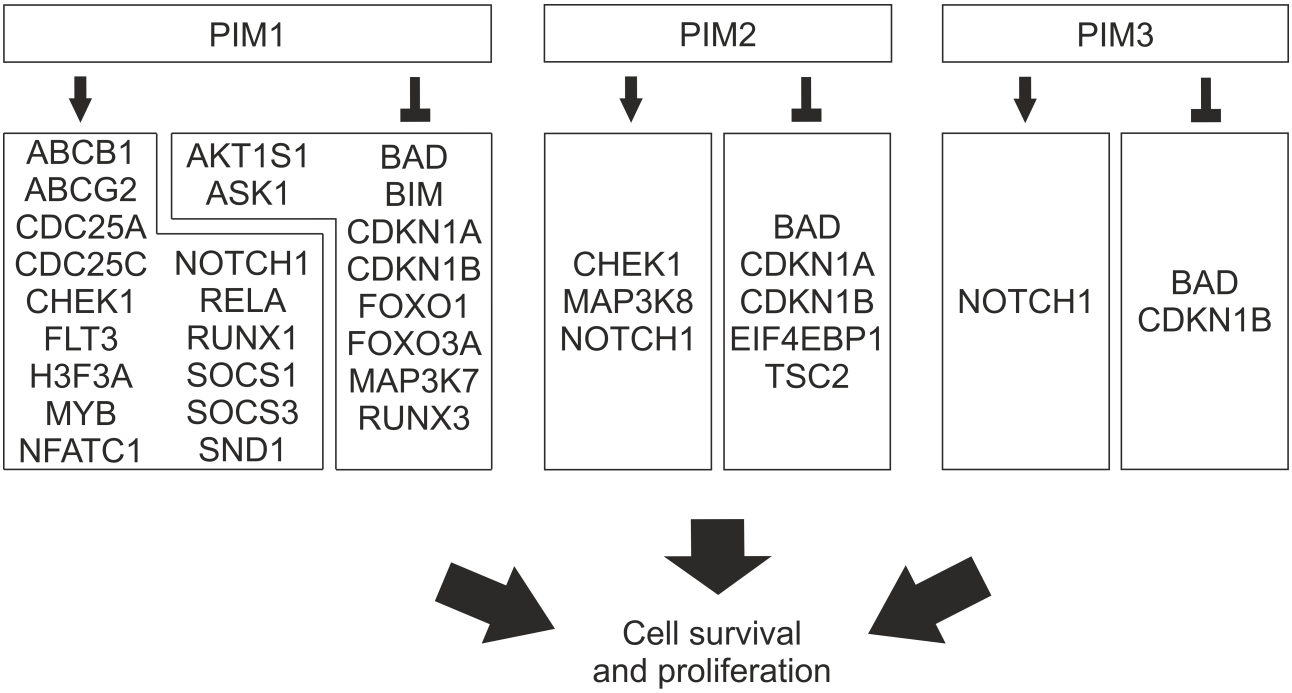
Figure 1.


PIM1
PIM2
PIM3
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ABCG2
CDC25A
CDC25C
CHEK1
FLT3
H3F3A
MYB
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AKT1S1
ASK1
NOTCH1
RELA
RUNX1
SOCS1
SOCS3
SND1
BAD
BIM
CDKN1A
CDKN1B
FOXO1
FOXO3A
MAP3K7
RUNX3
CHEK1
MAP3K8
NOTCH1
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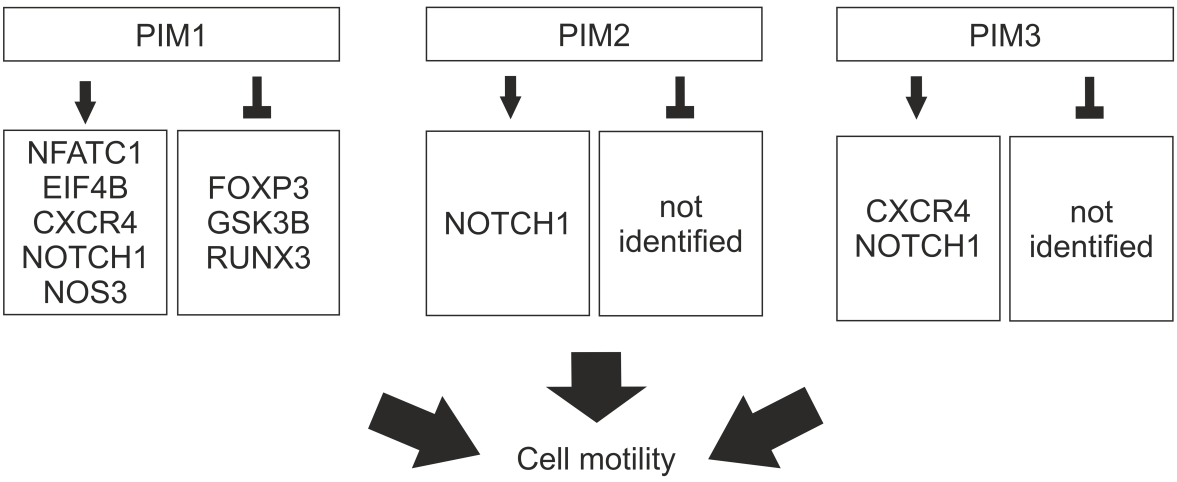
NOTCH1
BAD
CDKN1B
Cell survival
and proliferation

Figure 2.

a



b

MOUSE MODEL	*CANCER*	*PIM MODIFICATION*		*STUDY TARGET*
Carcinogenic induction	Sarcoma	PIM1/2/3 KO	⊣	Bone invasion
Subcutaneous	Pancreatic cancer	PIM3 (up)	→	Angiogenesis
Orthotopic	Pancreatic cancer	PIM3 (up)	→	Angiogenesis
	Prostate cancer	PIM1 (up) or PIM3 (up)	→	Angiogenesis Metastases
		pan-PIM inhibitor DHPCC-9	⊣	Angiogenesis Metastases
	Liver cancer	PIM1 KO	⊣	Metastases
Tail vein injection	Melanoma	PIM1-targeting miR-542-3p	⊣	Metastases
Genetic induction	Prostate cancer	pan-PIM inhibitor CX-6258*	⊣	Invasive lesions
Patient-derived xenograft	Prostate cancer	pan-PIM inhibitor CX-6258*	⊣	Proliferation of metastatic tissue

Table 1.

Kinase	Tissue	Association of high PIM protein levels with clinical outcome			Reference(s)
		Tumor size/ stage	*Metastases*	*Poor survival*	
PIM1	Bone cancer	-	+	+	(Liao et al., 2016; Mou et al., 2016)
PIM1	Brain tumor	-	-	+	(Herzog et al., 2015)
PIM1	Breast cancer	+	+	+	(Malinen et al., 2013; Brasó-Maristany et al., 2016; Horiuchi et al., 2016;)
PIM3	Breast cancer	+	-	-	(Ling et al., 2014)
PIM3	Colorectal cancer	-	+	+	(Zhou et al., 2016)
PIM1	Esophageal cancer	+	+	+	(Liu et al., 2010)
PIM1	Gastric cancer	+	+	+	(Warnecke-Eberz et al., 2009)
PIM3	Gastric cancer	-	-	+	(Zheng et al., 2008)
PIM1	Head and neck cancer	-	+	+	(Peltola et al., 2009; Tanaka et al., 2009)
PIM1	Lung cancer	+	+	+	Pang et al., 2014; Jiang et al., 2016)
PIM3	Ovarian cancer	+	+	-	(Zhuang et al., 2015)
PIM1	Pancreatic cancer	-	-	+	(Xu et al., 2016b)
PIM3	Pancreatic cancer	+	+	+	(Chang et al., 2016)
PIM1	Prostate cancer	+	-	-	(Valdman et al., 2004; Cibull et al., 2006, van der Poel et al., 2010)
PIM3	Prostate cancer	+	+	+	(Qu et al., 2016)
PIM1	Salivary gland cancer	+	-	+	(Zhu et al., 2014)

Table 2.

Kinase	Tissue	Modification of PIM expression levels or kinase activity			Reference(s)
		up	*down*	*inhibition*	
PIM1	B cell leukemia	-	x	K00135	(Decker et al., 2014)
PIM1	B cell lymphoma	-	-	K00486	(Brault et al., 2012)
PIM1, 2	Breast carcinoma	-	x	-	(Uddin et al., 2015)
PIM1, 3	Glioma	x	x	-	(Selmi et al., 2012, Deng et al., 2016)
PIM1-3	Head and neck squamocellular carcinoma	-	x	DHPCC-9, BA-1a	(Tanaka et al., 2009; Santio et al., 2010; Kiriazis et al., 2013)
PIM1	Hepatocellular carcinoma	-	x	-	(Leung et al., 2015)
PIM1	Melanoma	x	x	-	(Rang et al., 2016)
PIM1	Mesenchymal stem cells	-	-	Quercetagetin	(Zhang et al., 2016)
PIM1, 3	Nasopharygeal carcinoma	-	x	Quercetagetin	(Jie et al., 2012; Ai et al., 2016)
PIM1	Non-small-cell lung cancer	-	x	-	(Pang et al., 2014)
PIM1	Osteosarcoma	x	x	SMI-4a	(Liao et al., 2016; Mou et al., 2016)
PIM1, 3	Ovarian carcinoma	x	-	-	(Zhuang et al., 2015)
PIM1, 3	Pancreatic adenocarcinoma	-	x	-	(Xu et al., 2011, 2013; Chang et al., 2016;)
PIM1-3	Prostate carcinoma	x	x	DHPCC-9, BA-1a	(Santio et al., 2010; Kiriazis et al., 2013; Cen et al., 2014)
PIM1	Salivary gland carcinoma	x	-	SGI-1776	(Liu et al., 2015, Hou et al. 2017)
PIM1	T cell leukemia	-	x	K00486	(Grundler et al., 2009)
PIM1, 3	Vascular endothelial cells	x	x	SMI-4a	(Zippo et al., 2004; Zhang et al., 2009; Chen et al., 2016)