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The Multiple Roles of the IAP Super-family in Cancer

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Abstract:

The Inhibitor of Apoptosis proteins (IAPs) are a family of proteins that are mainly known for their anti-apoptotic activity and ability to directly bind and inhibit caspases. Recent research has however revealed that they have extensive roles in governing numerous other cellular processes. IAPs are known to modulate ubiquitin (Ub)-dependent signaling pathways through their E3 ligase activity and influence activation of nuclear factor κ B (NF κ B). In this review, we discuss the involvement of IAPs in individual hallmarks of cancer and the current status of therapies targeting these critical proteins.

Keywords: Inhibitors of apoptosis, hallmarks of cancer, cancer therapy, molecular targets

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Abbreviations:

BIR: Baculovirus IAP repeat

cIAP1: cellular inhibitor of apoptosis protein 1

cIAP2: cellular inhibitor of apoptosis protein 2

CPC: Chromosomal passenger complex

DIABLO: Direct IAP binding protein with low pI

DFS: Disease-free survival

E2: Ubiquitin conjugating enzymes

ECM: Extracellular matrix

EMT: epithelial to mesenchymal transition

FAK: Focal adhesion kinase

HIF: Hypoxia inducible factors

hTERT: Human telomerase reverse transcriptase

IAP: Inhibitors of apoptosis

IBM: IAP-binding motif

IKK: I κ B kinase

NAIP: Neuronal apoptosis inhibitor protein

NF κ B: Nuclear factor κ B

NIK: NF κ B inducing kinase

OS: Overall survival

PFS: Progression-free survival

RFS: Recurrence-free survival

RIPK1: Receptor-interacting serine/threonine-protein kinase 1

SMAC: Second Mitochondria-derived Activator of Caspases

TNFR: Tumor necrosis factor receptor

TNF α : Tumor necrosis factor α

TRAF: Tumor Necrosis Factor Receptor Associated Factor 2

Ub: Ubiquitin

VEGF: Vascular endothelial growth factor

XIAP: X-Linked inhibitor of apoptosis protein

1. Introduction:

The cells of multi-cellular organisms have the ability to activate an intrinsic cell suicide machinery associated with specific biochemical and morphological changes, termed apoptosis. This programmed cell death process is tightly regulated, and abnormalities in the apoptotic pathway have been associated with various human diseases such as cancer, neurodegeneration and autoimmune disease (Steller, 1995).

Inhibitor of Apoptosis Proteins (IAPs) are a family of proteins that regulate apoptosis by inhibiting caspases, which are cysteine-dependent aspartate-directed proteases that serve as the effector arm of this process. Initially, this was considered to be their primary function; however, recent studies have indicated that they have a number of different roles in other cellular processes. Various ubiquitin (Ub)-dependent signaling events that regulate important transcription factors responsible for the expression of genes involved in cell migration, survival and inflammation are now known to be affected by various members of the IAP family (Gyrd-Hansen & Meier, 2010). Moreover, many of these processes play roles in cancer initiation, metastasis and progression (Hunter, LaCasse, & Korneluk, 2007; LaCasse, et al., 2008).

In this review, we discuss the broad range of functions of the IAPs in cancer development by assessing their roles in the various hallmarks of cancer. We also briefly discuss the most extensively studied means of targeting these proteins.

1.1 Discovery of the IAP family of proteins:

The first IAP genes identified were from the baculovirus *Cydia pomonella* granulosis virus (CpIAP) and *Orgyia pseudosugata* nuclear polyhedrosis virus (OpIAP) in a complementary assay looking to identify additional genes which had the potential to inhibit apoptosis in a line deficient in p35, a pan-caspase inhibitor. These genes were found to have no sequence homology to p35 but contained a zinc finger-like motif which is also found in other genes regulating apoptosis (Birnbaum, Clem, & Miller, 1994; Crook, Clem, & Miller, 1993). The first mammalian IAP to be discovered was the neuronal apoptosis inhibitor protein (NAIP). This was after several studies showing inhibition of apoptosis by the baculovirus IAP in

various different species were published, raising the possibility of the protein having cellular homologs (Deveraux & Reed, 1999; Verhagen, Coulson, & Vaux, 2001). NAIP was identified during a positional cloning study that aimed to identify genes involved in spinal muscular atrophies (SMAs) as these disorders are characterized by the presence of swollen or chromatolysed motor neuron cells resembling the morphology of cells that have undergone programmed cell death or apoptosis (Duckett, et al., 1996; Roy, et al., 1995). Since the discovery of these IAPs, other members have been identified which include X-linked IAP (*XIAP*); identified in a search looking at sequences homologous to the baculovirus IAP protein CplAP and in an effort to find other human genes encoding IAP-like proteins. The XIAP sequence was then used to find other homologous human IAP genes and resulted in the discovery of baculoviral IAP repeat containing 2 (*BIRC2* or cellular IAP1 (cIAP1)) and baculoviral IAP repeat containing 3 (*BIRC3* or cellular IAP2 (cIAP2))(Listen, et al., 1996).

Tumor necrosis factor alpha (TNF α) is an inflammatory cytokine that mediates its effects through two distinct cell surface receptors, Tumor necrosis factor receptor 1 and 2 (TNFR1 and TNFR2). TNFR1 has been known to be involved in TNF α -mediated apoptosis, with cIAP1 and cIAP2 purified and cloned as part of studies looking for proteins associated with these receptor complexes (Rothe, Pan, Henzel, Ayres, & Goeddel, 1995). The human IAP family has subsequently expanded to include the following members: baculoviral IAP repeat containing 8 (*BIRC8* or ILP-1/testis specific IAP (Ts-IAP)), which was discovered during the mapping and genomic characterization of XIAP as a testis-specific transcript (Lagacé, et al., 2001); Baculoviral IAP Repeat Containing 5 (*BIRC5* or Survivin), which was identified by examining genes hybridizing to the effector cell protease receptor (EPR1) (Ambrosini, Adida, & Altieri, 1997); and Baculoviral IAP Repeat Containing 6 (*BIRC6* or BRUCE/Apollon) (Zhihong Chen, et al., 1999) and baculoviral IAP repeat containing 7 (*BIRC7* or ML-IAP/Livin) (Kasof & Gomes, 2001), both of which were identified through various homology searches. The IAP family of proteins now has 8 members comprising of NAIP, XIAP, cIAP1, cIAP2, Ts-IAP, Survivin, Apollon and Livin.

1.2 Structure of the IAP family of proteins:

The defining structural feature of the IAP family of proteins is the baculovirus IAP repeat (BIR) domain. These BIRs are around 70 residues long and are zinc binding domains that facilitate protein-protein interactions. The eight mammalian IAPs contain one to three copies of this domain (Fig 1). BIRs possess certain common features which include the presence of three conserved cysteine residues and one histidine residue which coordinate a zinc ion that is required to stabilize the BIR fold (Srinivasula & Ashwell, 2008). There are two types of BIR domain, type I and type II, and these are grouped on the basis of the presence of a deep peptide binding groove. The Type II BIRs contain a distinctive hydrophobic cleft through which they bind to specific N-terminal IAP binding motifs (IBMs) present on their interaction partners, caspases and IAP antagonists (Silke & Meier, 2013; G. Wu, et al., 2000). The type I BIRs on the other hand do not have a deep peptide binding groove, but instead have a shallow pocket and so do not interact with caspases or IAP antagonists. They are, however, known to interact with different target proteins, with the type I BIR of XIAP having been reported to directly bind TAB1, an upstream adaptor of the Transforming Growth Factor- β activated kinase 1 (TAK1) (M. Lu, et al., 2007), whereas the type I BIRs of cIAP1 and cIAP2 interact with Tumor Necrosis Factor Receptor Associated Factor 2 (TRAF2), which is an adaptor protein that facilitates signal transduction from the TNF receptor family (T. Samuel, et al., 2006).

In addition to the BIR domains, IAPs also contain a carboxy-terminal RING finger domain (Fig 1). This domain enables IAPs to recruit ubiquitin conjugating enzymes (E2) through which ubiquitin can be transferred onto lysine residues of target proteins. Proteins are often poly-ubiquitinated, with addition of further ubiquitin moieties to lysine residues present in ubiquitin itself. Depending on the lysine residues to which the incoming ubiquitin is conjugated, poly-ubiquitinated chains with different topologies can be created that determine the fate of the target protein, with ubiquitin chains joined via lysine 48 (K48) residues of ubiquitin recognized by the proteasome and typically targeted for degradation (Vaux & Silke, 2005). However, degradation is not the only outcome for poly-ubiquitinated proteins; for example, K63-linked poly-ubiquitin chains can instead result in modification of biological activity or sub-cellular

localization of the target protein (Komander & Rape, 2012; Ni, Li, & Zou, 2005). The RING domain of IAPs, such as cIAP1, have been shown to downregulate protein levels of other RING domain-bearing IAP family members by targeting them for degradation by both ubiquitin-dependent and -independent pathways (Cheung, Plenchette, Kern, Mahoney, & Korneluk, 2008). With respect to ubiquitination, IAPs also contain a ubiquitin-associated domain (UBA) and a ubiquitin conjugating domain (UBC). Apollon is the only member of the human IAP family of proteins containing a UBC domain. This motif can function as ubiquitin conjugating enzyme (E2), allowing it to transfer ubiquitin to substrates directly (Bartke, Pohl, Pyrowolakis, & Jentsch, 2004). XIAP, cIAP1, cIAP2 and Ts-IAP can bind to polyubiquitylated proteins and substrates through their UBA domains (Gyrd-Hansen, et al., 2008).

cIAP1 and cIAP2 also contain a caspase recruitment domain (CARD), a domain found in procaspase-9 and APAF-1, which is important for formation of the caspase-9-activating Apoptosome (Li, et al., 2017). The exact function of the CARD in cIAP1/2 is not fully elucidated, but it has been suggested that it plays a role in stabilizing the cIAP1 protein by inhibiting auto-ubiquitination by the RING domain (Oetjen & Duckett, 2011). IAPs thus have structurally varied features that confer on them distinct functions by allowing them to interact with important proteins and thereby regulate important signaling pathways.

1.3 Expression of IAP proteins in cancer and correlation with prognosis:

Deregulation of IAPs at gene, mRNA and protein levels due to genetic aberrations, changes in mRNA or protein expression and loss of endogenous inhibitors, such as Second Mitochondria-derived Activator of Caspases (SMAC), have been reported in many different cancer types and correlated with treatment response and prognosis. Tamm *et al.* studied the expression of IAPs in a panel of 60 cancer cell lines from 9 different cancer types and found that most of the cell lines expressed XIAP and cIAP1; however, cIAP2 was not easily detected at the protein level in the majority of the cell lines. Notably, mRNA levels did not correlate with protein levels, suggesting posttranscriptional regulation is important for these proteins

(Tamm, et al., 2000). Further exploration in clinical samples showed that IAPs were expressed in most cancer types, leading to more work being done to study their association with survival and response to therapy, as discussed below and summarized in Table 1.

Chromosomal aberrations in the 11q21-q23 regions which harbors the cIAP1 (*BIRC2*) and cIAP2 (*BIRC3*) genes have been reported in different cancer types. Imoto *et al.* found this region to be amplified in esophageal squamous cell carcinoma-derived cell lines and went on to identify the target for amplification at this region. They found that only cIAP1 was consistently over-amplified in the cell lines and also found it to be overexpressed in the primary tumors from which these cell lines were derived (Imoto, et al., 2001). Similar results identifying cIAP1 as the candidate proto-oncogene within this amplicon have been reported in pancreatic (Bashyam, et al., 2005), liver (Zender, et al., 2006), lung (Dai, et al., 2003) and other cancers. This includes cervical squamous cell carcinoma (CSCCs), in which amplification and overexpression of cIAP1 has been shown to be associated with resistance to radiation-induced cell death in cell lines, and it was also found to contribute to poorer overall survival (OS) and recurrence free survival (RFS) (Imoto, et al., 2002). In addition to 11q chromosomal aberrations, the BIR domains of cIAP2 are also involved in the generation of an oncoprotein in mucosa-associated lymphoid tissue lymphoma which involves fusion of the N-terminus of cIAP2 with mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1). This fusion protein constitutively activates the NF- κ B signaling pathway and is associated with advanced stage disease, particularly in cases of gastric MALT lymphomas, (Rosebeck, et al., 2011).

In acute myeloid leukemia (AML), a three gene expression signature which included cIAP2 was identified that could predict overall survival in newly diagnosed patients (Hess, et al., 2007). However, there exists contradicting reports on the association of the IAP family member proteins with prognosis in AML (Tamm, et al., 2000; Tamm, et al., 2004 Carter, et al., 2003). Similar contradicting reports also exist for Survivin (Carter, et al., 2003; A. Pluta, et al., 2015) and therefore further studies are required in order to better understand the prognostic importance of the IAP family in AML. Expression/function of the IAP family proteins are also deregulated in other hematological malignancies (S Fulda, 2009).

In renal cell carcinoma (RCC), higher XIAP expression levels were reported to be correlated with advancing tumor stage, dedifferentiation and grade of cancer. (Mizutani, et al., 2007; Ramp, et al., 2004). Notably, patients with bladder cancers expressing high levels of XIAP had significantly lower recurrence-free survival rates (p value = 0.0015), and this could also independently predict recurrence of non-muscular invasive bladder cancer (HR: 3.46, 95% CI: 1.37–7.97, p value = 0.004) (Ming, Tao, Yin, & Na, 2007). Another study in bladder cancer looked at cIAP1/2 expression levels and found similar results (X. Che, et al., 2012). In addition to these findings, Chen *et al.* reported that RFS was significantly reduced in the patient group whose bladder cancers showed high expression and of cIAP1, 2 and XIAP (low IAP expression: mean RFS 39.2 months versus high IAP expression group: mean RFS 26.6 months, p value = 0.001) (X. Chen, et al., 2013). They also found a positive correlation between nuclear cIAP1 and Survivin expression, with high expression of both these IAPs correlating with shorter RFS times (low cIAP+ Sur: mean RFS 36.4 months versus high cIAP+ sur: mean RFS 24.4 months, p value= 0.003) (X. Chen, et al., 2013). Furthermore, , a systematic review also showed that Survivin expression correlates with worse prognosis in bladder cancer (OS: pooled HR, 1.53; 95% CI, 1.02–2.29) and (RFS: pooled HR, 1.81; 95% CI, 1.30–2.52) and (PFS: pooled HR, 2.12; 95% CI, 1.60–2.82) (Jeon, Kim, Kwak, Kim, & Ku, 2013).

Interestingly, in breast cancer, one study specifically reported that nuclear XIAP expression and not cytoplasmic expression correlated with shortened overall survival (Y. Zhang, et al., 2011). Another study showed that nuclear Survivin expression could independently predict outcome in terms of both relapse-free and overall survival (Kennedy, et al., 2003). Pluta *et al.* found that tumor XIAP and Survivin expression were associated with more advanced breast (P. Pluta, et al., 2015). Interestingly, in non-small cell lung cancer (NSCLC), patients with tumors expressing higher XIAP had longer overall survival as compared to patients with tumors expressing lower levels (60 months versus 24 months of median survival, p value=0.01) (C. G. Ferreira, et al., 2001). In colorectal cancer (CRC), higher XIAP expression correlated with tumor differentiation, venous invasion and Dukes staging; moreover, XIAP expression was found to be an independent prognostic factor (HR: 2.730; 95% CI: 1.226–5.445; p value= 0.0206) (Xiang, Wen, Wang,

Chen, & Liu, 2009). High cIAP2 expression has also been associated with reduced survival rates in CRC (Krajewska, et al., 2005).

In pancreatic cancer (PC), upregulation of cIAP2 at the mRNA level correlated with resistance to various commonly used chemotherapeutic drugs cell line models (Lopes, Gangeswaran, McNeish, Wang, & Lemoine, 2007). Another study showed that cIAP2 expression levels increase during the progression of PC and that co-expression of cIAP1 and 2 is a predictor of worse prognosis (Esposito, et al., 2007). In addition to cIAP1/2, the XIAP negative regulator XAF1 (XIAP-associated factor 1) has been shown to influence prognosis of PC patients, with low XAF1 levels correlating with shorter survival times (J. Huang, et al., 2010). Similar results have been published in hepatocellular carcinoma (Augello, et al., 2009) and gastric cancer (Shibata, et al., 2007). Recent systematic reviews conducted on studying the association between XIAP levels and cancer prognosis in patients with different cancers have suggested that increased XIAP levels correlate with unfavorable prognosis (Gao, et al., 2019; S. Li, et al., 2019)

These studies indicate that the most widely studied IAPs: cIAP1 and 2 and XIAP are frequently overexpressed in a number of cancers and that their levels could help predict prognosis and clinical outcomes.

1.4 miRNA regulation of IAP expression

miRNAs have been reported to influence important cellular processes in cancer and can function either as tumor suppressors or oncogenes by regulating gene expression. Several miRNAs are involved in different stages of the apoptotic process and are known to regulate components of both the extrinsic and intrinsic apoptotic pathway (Y. Peng & Croce, 2016). Expression of various members of the IAP protein family are also reported to be affected by miRNAs, and this could be used to further develop the therapeutic targeting of these proteins in cancer. Xie Y *et al.* have shown that miRNA-24 targets XIAP in cancer cells and its overexpression overcomes apoptotic resistance resulting in TNF α induced cell death (Xie, et al., 2013). Furthermore, in ovarian cancer tissues and cell lines, expression of miRNA-137 has been

reported to correlate with XIAP expression and that expression of this miRNA resulted in increased sensitivity of ovarian cancer cells to cisplatin-induced apoptosis via its XIAP regulation activity (X. Li, et al., 2017). miRNA-200c and miRNA-212 have also been shown to downregulate XIAP in triple negative breast cancer (TNBC) (Ren, et al., 2014) and RCC (Gu, et al., 2017) respectively, resulting in reduced proliferation, migration and invasion of cancer cells. In RCC, miRNA-212 levels negatively correlated with XIAP and this also correlated with worse prognosis. Additionally, miRNA-based targeting of XIAP has been shown to regulate multi-drug resistance in retinoblastoma (Yang, Zhang, Lu, & Wang, 2020). Similar studies have been published on miRNAs targeting survivin (Jingcao Huang, Lyu, Wang, & Liu, 2015; Kogo, et al., 2015; C. Wang, Zheng, Shen, & Shi, 2012) livin (Ye, et al., 2013) as well as cIAP (Cao, et al., 2018), and this miRNA induced regulation of IAP expression plays an important role in controlling cancer cell proliferation, invasion and migration.

2. Roles of IAP in cancer: Connecting to the hallmarks of cancer:

Since the discovery of the IAP family of proteins, various studies have shown that they play many diverse roles in addition to regulating apoptosis. In the next sections, we will provide an outline of their different roles, connecting them to the hallmarks of cancer. This emphasizes the importance of targeting the IAP family of proteins in cancer therapy as they directly or indirectly regulate various pathways involved in tumor growth and progression.

2.1: Resisting cell death

One of the hallmarks of cancer is the ability of tumor cells to evade cell death, and the role of IAPs in this evasion has been extensively studied. Cells undergo apoptotic cell death via two major pathways, the extrinsic and intrinsic pathways, and both ultimately result in the activation of caspases. The extrinsic pathway involves interactions of specific transmembrane receptors with their respective ligands and the TRAIL-R1/R2 (DR4/5)/TRAIL receptor/ligand pairs are the most extensively studied. Ligand binding triggers homotypic interactions between the cytoplasmic death domains (DDs) of these receptors and the DDs of the cytoplasmic adaptor protein FADD. Via its death effector domain (DED), FADD then assembles the death inducing signaling complex (DISC), into which procaspase-8 is recruited and can be activated to initiate apoptotic signaling (Riley, Malik, Holohan, & Longley, 2015). In the intrinsic apoptotic pathway, various intracellular stimuli cause changes in the permeability of the outer mitochondrial membrane, resulting in the release of pro-apoptotic proteins like cytochrome c and SMAC. This then results in the activation of initiator caspase 9. Mitochondrial outer membrane permeabilisation (MOMP) is controlled by the members of the BCL2 family that include both pro- and anti-apoptotic proteins, such as Bcl-2, Bcl-XL, Mcl-1 and A1, which are anti-apoptotic, and Bax, Bak, Bid, Bad, Bim, Noxa and Puma, which are the pro-apoptotic members (Wilson, Johnston, & Longley, 2009). The two pathways converge as Bid is cleaved by caspase-8 and result in activation of the effector caspases-3, -6 and -7, which then go on to cleave specific target proteins that effect apoptosis (Elmore, 2007).

IAPs as direct inhibitors of caspases:

The activity of both initiator and effector caspases are regulated by IAPs. The first studies on the mechanism of apoptosis inhibition by IAPs came in 1997 when Devereux *et al.* published reports where they found that XIAP can regulate caspase-3 processing following caspase-8 activation. Thus, it was shown that XIAP could inhibit the extrinsic apoptotic signaling pathway by targeting the downstream effector caspases, as opposed to directly affecting caspase-8 activity. On the other hand, IAPs were also shown to inhibit the initiator caspase-9 in the intrinsic apoptotic pathway, thereby regulating the proteolytic activation of procaspases-3 and -7 (Deveraux, et al., 1998; Deveraux, Takahashi, Salvesen, & Reed, 1997). Thus, XIAP can control both initiator caspase-9 and effector caspases-3 and -7 activities directly in order to regulate the extrinsic and intrinsic apoptotic pathways.

XIAP interacts with caspase-9 through its BIR3 domain, with its C-terminus binding to the dimer interface of caspase-9. This prevents the conformational changes needed for the generation of the active catalytic pocket, thereby inhibiting its activity (Berthelet & Dubrez, 2013). In the case of the interaction of XIAP with caspases-3/7, the residues in the linker region between BIR1 and BIR2 are involved and bind to the active site pocket of the caspases. The BIR2 domain of XIAP makes additional contacts with an IBM motif on the effector caspases thereby enhancing XIAP's interaction with the caspase (Riedl, et al., 2001; C. Sun, et al., 1999). The role of the RING domain in inhibiting caspases has been studied in mouse models. Schile *et al.* created a mouse model with a truncated RING domain and observed that cells expressing the RING mutant had significantly higher caspase activity and were sensitized to TNF α -induced apoptosis, indicating that the BIR domain alone is not enough to inhibit caspases (Schile, Garcia-Fernandez, & Steller, 2008).

cIAP1 and 2 have been reported to bind to caspases, but do not have the ability to directly inhibit them, making XIAP the only member to act as a direct inhibitor of caspases (Eckelman & Salvesen, 2006; Eckelman, Salvesen, & Scott, 2006).

Role of IAPs in regulating cell survival and inflammation via NFκB signaling pathway:

In addition to directly inhibiting caspases, IAPs play a very important role in regulating the NFκB signaling pathway, which regulates expression of many different target genes that promote cell proliferation as well as regulate apoptosis, invasion and metastasis (Park & Hong, 2016; Xia, et al., 2018). NFκB is a family of hetero- and homo-dimeric transcription factors that consists of the components RELA, RELB and CREL along with the precursor ankyrin containing proteins NFκB1 (p105) and NFκB2 (p100). The latter are processed via the proteasome to generate p50 and p52 respectively. p50/52 do not contain any transactivating domains and therefore depend on their interaction with RELA, RELB and CREL to drive transcription. There are different ways in which NFκB can be activated, classified into two distinct pathways: canonical or non-canonical pathway, both of which are regulated by ubiquitination-dependent signaling cascades (J. Chen & Chen, 2013; Perkins, 2007).

Canonical NFκB pathway: The most studied mechanism of activation of the NFκB pathway is via binding of TNFα to its receptor TNFR1, resulting in the recruitment of TRADD, TRAF5, TRAF2, cIAP1, cIAP2 and Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) to form “Complex-I”. cIAP, through its E3 ligase activity, then conjugates components of this complex with ubiquitin. One of the components that is Ub conjugated by cIAP is RIPK1 (Bertrand, et al., 2008), which then recruits the Linear Ub chain assembly complex (LUBAC) that is composed of HOIL/HOIP/Sharpin, the kinases TAK1/TAB2/TAB3 and the IκB kinase (IKK) complex composed of IKKα/IKKβ/NEMO. LUBAC then adds Ub chains to NEMO which undergoes a conformational change resulting in the autophosphorylation of IKK. Activated IKK then phosphorylates inhibitor of κB (IκB) which undergoes Ub-dependent proteasomal degradation allowing NFκB to translocate to the nucleus leading to the expression of its target genes (Behrends & Harper, 2011; Darding & Meier, 2012). In the absence of cIAPs, TNFR1 stimulation leads to the formation of a different complex that is mainly composed of RIPK1, FADD and caspase-8 called Complex-II. This complex is similar to the DISC and can activate caspase-8, leading to apoptosis (Petersen, et al., 2007; Vince, et al., 2007). Breakdown of Complex-I following de-ubiquitination of RIPK1 by the de-ubiquitinating enzymes (DUBs)

Cylindromatosis (CYLD), Cezanne and A20 results in the formation of a secondary complex termed Complex-IIA, which consists of TRADD instead of RIPK1, FADD and caspase-8. Complex-IIA, Complex-IIB and the DISC also contain the caspase-8 paralog and regulator c-FLIP (cellular Fas-associated death domain (FADD)-like interleukin-1 β -converting enzyme (FLICE/caspase-8)-inhibitory protein). Depending on its expression levels, FLIP can inhibit caspase-8 activation in these complexes, thereby promoting cell survival. As the FLIP gene (*CFLAR*) is an NF κ B target, Complex-I can modulate the signaling outcomes from Complex-IIA/B. This includes a non-apoptotic form of cell death termed necroptosis, which is mediated by RIPK1 and RIPK3 (Tsuchiya, Nakabayashi, & Nakano, 2015). The long splice form of FLIP, FLIP(L), and caspase-8 can form an enzymatically active heterodimer in Complex-IIA/B, which cleaves RIPK1, thereby preventing the induction of necroptosis, a much more pro-inflammatory form of cell death than apoptosis (Humphreys, Espona-Fiedler, & Longley, 2018; Micheau & Tschopp, 2003; L. Wang, Du, & Wang, 2008). The ripoptosome is another complex with the same composition as Complex-IIB but formed in the absence of TNFR1 stimulation, which is induced in response to cIAP1/2 depletion following high dose chemotherapy or treatment with IAP antagonists (Imre, Larisch, & Rajalingam, 2011; Tenev, et al., 2011). (Fig 2a)

Thus, IAPs play a pivotal part in death receptor signaling pathways through their role in ubiquitination of components of complex I leading to recruitment of LUBAC and subsequent activation of NF κ B and also by negatively regulating the formation of the RIPK1-dependent ripoptosome complex which can promote cell death via apoptosis and necroptosis.

Non-canonical NF κ B signaling pathway: Non-canonical NF κ B activation occurs mostly in response to stimulation by TNF family receptors such as CD40L, B cell activating factor (BAFF) and TWEAK. It depends on the proteasomal processing of NF κ B2 to its p52 form which is regulated by NF κ B-inducing kinase (NIK) (S. C. Sun, 2011, 2017). In resting cells, NIK is constitutively degraded through the formation of a Ub ligase-

dependent complex that consists of TRAF3-TRAF2 and cIAP1 and/or 2. TRAF3 directly binds NIK, but studies have shown that the cIAP-mediated Ub ligase activity is needed for the conjugation of degradative polyubiquitinated chains linked to NIK (Zarnegar, et al., 2008). Ligand binding to CD40L/BAFF/TWEAK results in TRAF3 being targeted for proteasomal degradation following ubiquitination by cIAP. In the absence of TRAF3, NIK can no longer be recruited to the cIAP/TRAF complex, therefore its levels increase. Stabilized NIK phosphorylates IKK β , which in turn phosphorylates p100/NF κ B2 triggering the latter's proteasomal processing to p52. Processed p52 complexes with RelB and stimulates expression of the heterodimer's (Fig 2b). Thus, in contrast to their role in the canonical NF κ B pathway, IAPs negatively regulate the non-canonical NF κ B pathway by preventing the accumulation of NIK.

Role of IAPs in regulating autophagy:

Autophagy is an important catabolic process that normally functions at low basal levels, but can be induced in response to stressful conditions, with the most common being nutrient deficiency. It functions by the formation of autophagosomes that capture degraded cellular components and fuse with lysosomes to recycle them. Autophagy is a double-edged sword as it is a tumor suppressive process, but can promote therapy resistance by enabling tumor cells to survive chemotherapy-induced stress (Das, Shrivage, & Baehrecke, 2012; Yun & Lee, 2018). Recent evidence points to the role of IAPs in regulating autophagy, with XIAP, cIAP2 and BRUCE reported to play a role in regulating autophagosome and lysosome fusion (Ebner, et al., 2018; Gradzka, et al., 2018; Pötsch, et al., 2018). In addition, XIAP and cIAP1 induce autophagy by upregulating the expression of Beclin-1, which is an important modulator of autophagy. The resistance to chemotherapy observed in cancers overexpressing XIAP and cIAP1 has been related, at least in part, to induction of autophagy caused by Beclin-1 upregulation (Lin, et al., 2015).

In contrast to these reports suggesting a positive role of IAPs in mediating autophagy, IAPs are also known to inhibit autophagy. Che *et al.* reported that loss of BRUCE induces autophagy and

increases autophagic activity by lowering cellular energy levels (L. Che, et al., 2019). XIAP has also been shown to inhibit autophagy via its ubiquitin E3 ligase activity by interacting with MDM2, which is a negative regulator of the proto-oncoprotein p53 (X. Huang, Wu, Mei, & Wu, 2013). It appears therefore that IAPs can inhibit or induce autophagy depending on the specific IAP and cellular context.

IAPs and cancer stem cells:

Dysregulation of various signaling pathways play an important role in resistance to cell death in cancer stem cells (CSCs). These CSCs are believed to be one of the main reasons for recurrence in many different cancer types and this is due to their treatment-resistant nature. The IAP family of proteins have been implicated in contributing to the resistance mechanisms seen in CSCs (A. R. Safa, 2016; Ahmad R Safa, 2019). Survivin is one of the most studied IAP family member in this context. Yu *et al.* have reported that breast cancer stem cells show elevated expression of survivin, and this was associated with multi-drug resistance and reduced apoptosis (Yu, Ou, Wang, Jialielihan, & Liu, 2015). Survivin expression is also reported to play a role in 5FU resistance in CD133+ colon cancer stem cells (M.-R. Lee, Ji, Mia-Jan, & Cho, 2015). Another study looking to identify therapeutic targets to overcome chemoresistance in colon cancer stem cells identified BRUCE as a target using a proteomics approach. They found that BRUCE was one of the top upregulated proteins in the stem cell population as compared to the differentiated cells (Van Houdt, et al., 2011). Interestingly, Siddarth *et al.* report the presence of a population of quiescent-breast cancer stem cells in a cell line model of metastasis. This population of adherent, pre-metastatic stem cells were found to express survivin which was important in the epithelial to mesenchymal transition of the cells and favored invasion and metastasis (Siddharth, Das, Nayak, & Kundu, 2016). Also, Tchoghandjian *et al.* showed that targeting IAP proteins with their

respective antagonists, resulted in loss of stemness and differentiation of glioblastoma cancer stem-like cells which was via regulation of the NF κ B pathway. They also went on to show that targeting IAPs under hypoxic conditions resulted in loss of cell viability of the glioblastoma stem-like cells, reduced proliferation and increased apoptosis but did not affect stemness (Souberan, et al., 2019; Tchoghandjian, et al., 2014). This is particularly important as hypoxia is one of the many reasons for chemoresistance seen in cancer cells, and the fact that cancer stem cells in hypoxic regions could be targeted by IAP antagonists makes them clinically relevant targets. Further research is required to investigate the potential of targeting IAPs in cancer stem cells.

2.2 Activating Invasion & Metastasis

One of the striking features of cancer cells is their ability to migrate from the primary site of tumor formation to distant sites through a process of invasion and metastasis. During tumorigenesis, cancer cells develop ability to alter their shape as well as their interactions with other cells and the extracellular matrix (ECM). This is now thought to involve an epithelial to mesenchymal transition (EMT) which results in increased expression of mesenchymal markers, such as N-cadherin, vimentin and fibronectin, that help invasion of surrounding tissues, a necessary first step of metastasis (Hanahan & Weinberg, 2011; Martin, Ye, Sanders, Lane, & Jiang, 2013). The IAP family of proteins have now been shown to be involved in various stages of cell migration, invasion and metastasis and this will be discussed further in the section below and is summarized in Fig 3.

Role of IAPs in regulating cell adhesion molecules:

Recent studies have highlighted the role of IAPs in the induction of the EMT process, with Jo *et al.* showing that cIAP2 promotes migration and invasion of TNBC cells by inducing EMT via activation of the AKT signaling pathway. They showed that silencing of cIAP2 resulted in reduced levels of mesenchymal

markers at both protein and mRNA level and also reduced the ability of breast cancer cells to migrate and invade the matrigel in wound-healing assays (Jo, et al., 2017). Similar results were found for Livin in promoting EMT in breast cancer cells *in vitro* by enhancing cell motility, invasiveness and metastasis (F. Li, et al., 2013). In addition to cIAP and Livin, XIAP and Survivin also reportedly play some role in the EMT process. Yi X *et al.* showed that simultaneous silencing of XIAP and Survivin in pancreatic cancer cells resulted in partial reversion of EMT, with increased expression of epithelial markers like E-cadherin and down-regulation of mesenchymal markers like Slug (Yi, Han, Li, Long, & Li, 2015). Survivin is also said to play a role in activating the EMT induced by insulin-like growth factor (IGF) in hepatocellular carcinoma (HCC) (F. Liu, et al., 2018).

Integrins are membrane receptors that link ECM components such as fibronectin and vitronectin to the actin cytoskeleton and various focal adhesion molecules, including Src and focal adhesion kinase (FAK). These molecules are responsible for regulating signals controlling cell adhesion, migration, survival and proliferation through phosphorylation of adherens junction components (Dubreux & Rajalingam, 2015). Recently, it has been reported that XIAP can interact with caveolin-1 and that this interaction is essential for endothelial cell survival (Kim, et al., 2008). Caveolin-1 is an integral membrane protein that regulates integrin-mediated cell adhesion. The authors have also shown that through its interaction with Caveolin-1, XIAP is recruited to the focal adhesion complex. This facilitates the joining of the integrin complex to the kinase FAK, which then results in FAK-mediated activation of ERK1/2, ultimately leading to increased endothelial cell survival and migration (Figure 3A) (Kim, et al., 2010). It has now been shown that XIAP regulates FAK activity by controlling FAK phosphorylation and that XIAP knockdown in endothelial cells resulted in decreased recruitment of FAK to the focal adhesion site, thereby affecting its interaction with Src. This in turn disrupted the activation of ERK1/2, thus inhibiting cell adhesion and migration (Ahn & Park, 2010). Since both Src and FAK play an important role in regulating the stability and availability of E-cadherin (Avizienyte & Frame, 2005; Pei, Lan, Chen, Ji, & Hua, 2017), XIAP-mediated regulation of FAK activity could also play a major role in epithelial cell migration and participate in the process of cancer cell

invasion and metastasis. Additionally, Mehrotra *et al.* demonstrated that XIAP in a complex with Survivin promotes invasiveness in breast carcinoma cells and is also responsible for metastatic dissemination *in vivo*. This XIAP/Survivin complex was shown to upregulate NF κ B-mediated transcription of fibronectin, resulting in the activation of β -integrins via autocrine/paracrine signaling which led to the phosphorylation and activation of cell motility kinases Src and FAK (Mehrotra, et al., 2010).

IAPs also appear to interact with various migration-associated pathways like ERK/MAPK, TGF β and, as discussed earlier, NF κ B. cRAF is a central member of the MAPK pathway, the increased activation of which activates ERK1/2, an important regulator of cell motility (Figure 3B). XIAP, cIAP1/2 and even ML-IAP have been shown to directly interact with cRAF. Dogan *et al.* have shown that both XIAP and cIAP1/2 directly bind to cRAF and that silencing of XIAP and cIAPs leads to stabilization of cRAF and increased cell migration (Dogan, et al., 2008). Similar findings have been published by Oberoi-Khanuja *et al.*, who reported a direct interaction between ML-IAP and cRAF. This binding results in cRAF being targeted for proteasomal degradation, with loss of ML-IAP resulting in increased MAPK activation and cell migration (Oberoi-Khanuja, Karreman, Larisch, Rapp, & Rajalingam, 2012).

In addition to cRAF and ERK/MAPK signaling, IAPs also modulate gene expression induced in response to TGF β , which regulates transcription of various EMT regulators and also controls actin cytoskeleton and fibronectin expression (Hao, Baker, & Ten Dijke, 2019). XIAP has been reported to act as a cofactor in TGF β signaling independent of its role in apoptosis by associating with members of the type I class of TGF β receptors (Birkey Reffey, Wurthner, Parks, Roberts, & Duckett, 2001). Also, in esophageal carcinoma cell lines, XIAP has been reported to enhance cell migration by activating TGF β , which then promotes EMT (Jin, Lu, Wang, Zhao, & Xue, 2019). As discussed previously, IAPs are now well-known regulators of the canonical and non-canonical NF κ B signaling, which is also known to play an important role in cancer cell migration/invasion by regulating Snail and other EMT regulators (Y. Wu & Zhou, 2010).

Role of IAPs in regulating RhoGTPases:

Tumor invasion and metastasis involves the remodeling of the actin cytoskeleton resulting in changes in cellular organization and in cell adhesion with the surrounding tissues. It involves extension of membrane protrusions in the cell front to enable interaction with the ECM and focal adhesion complexes. RhoGTPases are a family of proteins that are known to play an important role in regulation of cell shape and remodeling of the actin cytoskeleton during cell migration. RhoA, Rac and Cdc42 are the most studied members of this family of proteins (Haga & Ridley, 2016). Interaction of IAPs with RhoGTPases has been confirmed by various studies (Figure 3C). Oberoi *et al.* demonstrated that both XIAP and cIAP directly bind to the RhoGTPase member Rac1, resulting in its ubiquitination and proteasomal degradation; moreover, they found that downregulation of IAPs restores Rac1 levels, leading to enhanced cell migration and changes in cell morphology (Oberoi-Khanuja & Rajalingam, 2012; Oberoi, et al., 2012). RhoGDI regulates GDP-GTP nucleotide exchange and promotes actin cytoskeleton reorganization in fibroblast cells. Marivin *et al.* reported that cIAP1 directly interacts with Cdc42 and promotes its stabilization following TNF α stimulation (Marivin, et al., 2014). XIAP has also been shown to interact with RhoGDI and regulate cytoskeleton formation leading to increased cell motility (J. Liu, et al., 2011); subsequently, XIAP knockdown resulted in a reduction of β -actin polymerization, which was associated with decreased cell migration and invasion. This role of XIAP in regulating the cell motility is independent of its caspase regulatory activity but required the XIAP RING domain, although this was not found to be directly involved in interaction with RhoGDPI (J. Liu, et al., 2012). These studies show that IAPs are regulators of RhoGTPases and thereby play an important role in the cell migratory process.

2.3 Sustained proliferation:

Normal cells have a carefully regulated process of the release and production of growth factors that control the entry into and advancement through the various stages of the cell cycle. This ensures that

homeostasis is achieved and cell numbers are maintained at normal levels. Cancer cells are known to deregulate these signals, thereby becoming resistant to proliferation control mechanisms and acquire the ability to sustain chronic proliferation (Hanahan & Weinberg, 2011). They achieve this property by altering expression of various cell cycle proteins, constitutive activation of various signaling pathways and by interfering with the regulation and production of various growth factors (Feitelson, et al., 2015).

One of the most striking ways in which IAPs contribute to this sustained proliferation seen in cancerous cells is by being involved in the cell cycle process and Survivin the most studied IAP in this context. Survivin is reported to be a nuclear-cytoplasmic shuttling protein with a different role in each region. It contains a nuclear export sequence that enables this movement, with the cytoplasmic protein playing a role in suppressing apoptosis, as discussed earlier, while nuclear Survivin is reported to regulate proliferation (Colnaghi, Connell, Barrett, & Wheatley, 2006; Connell, Colnaghi, & Wheatley, 2008). The role of Survivin in cancer cell proliferation stems from its cell cycle specific regulation, and it is localized at different regions of the chromosome during the cell cycle (Altieri, 2004). Additionally, Survivin also forms an integral part of the chromosomal passenger complex (CPC). The CPC functions to ensure that the chromosomes are segregated properly during cytokinesis and is also responsible for establishing an orderly exit from mitosis. This is critical because dysfunctional mitotic exit or a defect in chromosome segregation can lead to genomic instability (Kitagawa & Lee, 2015; Ruchaud, Carmena, & Earnshaw, 2007b). The CPC is composed of 4 proteins with aurora B kinase being the enzymatic subunit and Survivin, inner centromere protein (INCEP) and borealin acting as its regulatory subunits. They all have interconnected roles and if either of them is dysfunctional, the others fail to localize and aurora B kinase does not function properly. Survivin is reported to serve as an interface between the centromere and the CPC and also mediates co-localization of the CPC to its specific mitotic structures (Ruchaud, Carmena, & Earnshaw, 2007a; Vader, Kauw, Medema, & Lens, 2006). Aurora kinases are also known to interact with the tumor suppressor protein p53 and phosphorylate it, leading to accelerated proteasomal degradation. Although, their

interactions with mutant p53 need to be studied further, the CPC definitely plays an important role in regulating cell proliferation (Gully, et al., 2012; Sasai, Treekitkarnmongkol, Kai, Katayama, & Sen, 2016).

In addition to being a part of the CPC, Survivin has also been reported to be involved with other cell cycle regulators. It is phosphorylated by cyclin dependent kinase-1 (CDK1), and this increases the affinity of Survivin for caspase-9 during cell division which could prevent apoptosis of dividing cancerous cells (Chandele, Prasad, Jagtap, Shukla, & Shastri, 2004; O'Connor, Grossman, et al., 2000; O'Connor, Schechner, et al., 2000). Cyclin dependent kinases (CDKs) are the key regulators of cell cycle progression and are controlled by several different processes. This involves binding of activating cyclin subunits and inhibitory INK4 and Cip1 subunits. p16^{INK4a} and p21^{WAF1/Cip1} belong to the family of CDK inhibitors that bind to CDK4/cyclin D1 and CDK2/cyclin E complexes and inhibit their activation and thereby play important role in G1 arrest (Satyanarayana & Kaldis, 2009; Sherr, Beach, & Shapiro, 2016). Suzuki *et al.* have also shown that overexpression of Survivin results in an accelerated shift to the S phase, accompanied by increased resistance to G1 arrest. This is via its interaction with the CDK4/p16^{INK4a} complex resulting in activation of CDK2/cyclin and phosphorylation of the retinoblastoma (Rb) protein. Rb phosphorylation is important in transversal through G1 and entry into the S phase of the cell cycle and results in inactivation of its proliferation suppressive function (Suzuki, et al., 2000). Similar results were shown by Ito *et al.* in HCC cell lines, with Survivin overexpression resulting in obliteration of the G1 checkpoint mediated by Survivin induced release of p21WAF1/Cip1 from CDK4 (Ito, et al., 2000). The fact that Survivin interacts with these complexes suggests that it could play an important role in sustaining cell proliferation seen in cancer cells.

The other members of the IAP family, cIAP1 and Livin have also been reported to play a role in cell cycle regulation. cIAP1 is now known to localize to the nucleus and modulate the cell cycle, with its overexpression associated with genomic defects and cell cycle aberration (Temesgen Samuel, et al., 2005). cIAP1 also plays a role in regulating E2F1 transcriptional activity when localized in the nucleus. E2F1 is a member of an important family of transcription factors that regulate cell differentiation and proliferation

resulting in expression of genes that are essential in cell cycle progression. The E3 ligase activity of cIAP1 aids in polyubiquitination of lysine residues that are associated with E2F1 transcriptional stability and activity (Cartier, et al., 2011; Glorian, et al., 2017). Livin is also reported to be involved in regulation of cell proliferation and transition to various stages of the cell cycle. Silencing of Livin results in inhibition of cell proliferation and cell cycle arrest at G0/G1 phase (LinLin Wang, Zheng, Tang, Li, & An, 2009; R. Wang, et al., 2008; Ye, et al., 2011) .

Cell proliferation is regulated by various molecular pathways whose constitutive activation results in uncontrollable tumor growth and division. Expression of various members of the IAP family are modulated by interacting with prominent cell signaling pathways. For example, Survivin is known to be critically modulated by interacting with heat-shock protein (hsp90), which is a chaperone protein responsible for maintaining the integrity of the cellular protein network; moreover, this interaction is known to promote tumor proliferation (Fortugno, et al., 2003; Kanwar, Kamalapuram, & Kanwar, 2011). Myc is an oncogene that is dysregulated in many cancers and has a central role in many aspects of the oncogenic process including cell proliferation. Xu *et al.* have demonstrated that cIAP1 is responsible for ubiquitination of the Myc repressor Mad1 leading to its proteasomal degradation. This reduction in Mad1 levels then resulted in Myc-mediated promotion of cell proliferation and transformation (L. Xu, et al., 2007).

Taken together, these studies show that IAPs can regulate tumor cell proliferation at different levels.

2.4: Enabling Replicative Immortality

In contrast to normal cells that undergo a finite number of cell division cycles before entering senescence or undergoing apoptosis, cancer cells acquire the ability to undergo unlimited proliferation; this is associated with aberrant expression of hTERT (human telomerase reverse transcriptase). hTERT maintains telomeres and prevents telomere shortening, which normally happens during each cell division, from triggering senescence/apoptosis. A number of reports

have correlated Survivin expression with hTERT expression in tumor tissues (Kleinschmidt-DeMasters, et al., 2003; Lam, Saleh, Smith, & Ho, 2008; Wellenhofer & Brustmann, 2012; Würfl, et al., 2002). Moreover, recent reports have shown that Survivin also regulates telomerase activity. Endoh *et al.* demonstrated that overexpression of Survivin enhanced hTERT expression in colon cancer cells. They then went on to show that Survivin increases hTERT expression by increasing the binding of specificity protein-1 (Sp1) and c-Myc to the hTERT promoter via phosphorylation of these transcription factors and this ultimately results in increased hTERT gene transcription (Endoh, Tsuji, Asanuma, Yagihashi, & Watanabe, 2005). Further research into this mechanism showed that Survivin phosphorylates Sp1 and c-MYC via its interaction with aurora B kinase (Furuya, Tsuji, Kobayashi, & Watanabe, 2009). Also, Yuan *et al.* reported that in non-transformed human fibroblasts transduced with hTERT, Survivin expression dramatically increased during immortalization, and this resulted in the cells being resistant to apoptosis (Yuan, et al., 2009). Moreover, they reported that expression of hTERT alone is not sufficient to induce immortalization and that increased expression of Survivin is also required.

2.5. Angiogenesis

Angiogenesis involves formation of new blood vessels from pre-existing ones, and vasculogenesis involves the generation of new endothelial cells which then go on to assemble into new blood vessels. Tumor cells are unable to grow in size, invade surrounding tissues and sustain growth if they fail to activate this process, which is one of the classical hallmarks of cancer (Yadav, Puri, Rastogi, Satpute, & Sharma, 2015). Various studies have shown that Survivin expression correlates with angiogenesis in different cancers (Kawasaki, et al., 2001; G.-H. Lee, et al., 2006; J. Li & Wu, 2006; Zhen, et al., 2005). In addition, co-expression of Survivin with the pro-angiogenic

factor VEGF correlated with cancer metastasis, stage and also prognosis in small-cell lung (P. Chen, et al., 2014), nasopharyngeal carcinoma (Y. H. Li, et al., 2008) and thyroid cancer (H. Y. Zhang, et al., 2009) and was also associated with tumor relapse in superficial bladder transitional cell carcinoma (Y. W. Sun, et al., 2013).

Two decades ago, it was reported that VEGF induces Survivin expression in vascular endothelial cells in a cell cycle dependent manner and that this facilitated angiogenesis (Tran, et al., 1999). Similar findings were also reported by Connor *et al.*, where they show that formation of three-dimensional vascular tubes *in vitro* in endothelial cells was associated with a marked induction of Survivin by VEGF. Also, *in vivo* studies have shown that Survivin was minimally expressed in the endothelium of normal skin, but was significantly upregulated during angiogenesis in newly formed blood vessels in granulation tissue (O'Connor, Schechner, et al., 2000). Meng *et al.* demonstrated that VEGFR signaling upregulated Survivin expression via the AKT/MDM2 pathway and that this was essential for tumor cell survival (Meng, et al., 2018). It has also been shown in glioma cells that Survivin can promote endothelial cell migration and proliferation and also increase microvessel density (a surrogate marker for tumor angiogenesis); moreover, this was attributed to Survivin-induced expression of angiogenic factors VEGF and FGF (P. Wang, et al., 2012). These findings are further supported by Li *et al.*, who found that in rat aortic endothelial cells, Survivin promoted tumor angiogenesis via enhanced tube and microvessel formation, which was accompanied by increased expression of VEGF (Z. Li, et al., 2016).

Studies in zebrafish have shown that Survivin knockdown greatly impairs the process of angiogenesis in embryos and leads to developmental defects; notably, VEGF was able to entirely rescue this phenotype (Delvaeye, et al., 2009; Ma, et al., 2007). Moreover, in neuroblastoma cells, Survivin knockdown combined with flavonoid treatment inhibited formation of the

angiogenic network *in vitro* and also resulted in downregulation of VEGF (Hossain, Banik, & Ray, 2012). Similar results were found in *in vitro* and *in vivo* studies in hepatocellular carcinoma (Shen, et al., 2014; G. C. Xu, et al., 2012) and in gastric cancer (Tu, et al., 2003), where Survivin inhibition greatly affected tumorigenicity and angiogenesis.

Role of IAPs in hypoxia and angiogenesis:

In cancer, tissue oxygenation is severely impaired because tumor cells rapidly outgrow their blood supply resulting in a condition known as hypoxia. A small sub-population of tumor cells develop the ability to survive in these hypoxic conditions and also simultaneously become more resistant to chemotherapy and radiotherapy (Muz, de la Puente, Azab, & Azab, 2015). Hypoxia inducible factors (HIFs) are a family of transcriptional complexes that are major sensors of hypoxia and regulate how cancer cells respond in this oxygen-deprived microenvironment. It is now established that there exists a close relationship between angiogenesis and hypoxia as HIF activation is an important stimulus for blood vessel growth. HIFs have been known to regulate various pro- and anti-angiogenic factors like VEGF (Hashimoto & Shibasaki, 2015; Krock, Skuli, & Simon, 2011; Lv, et al., 2017). In addition, HIFs have been reported to up-regulate expression of various IAP genes. Chen *et al.* studied the expression of HIF α and Survivin in non-small cell lung cancer and reported that HIF α binds to the promotor of the Survivin gene and increases its transcription (Y. Q. Chen, Zhao, & Li, 2009). Additionally, HIF α -mediated up-regulation of Survivin has been reported in cervical cancer (Bai, Ge, Lu, Qian, & Xu, 2013) and in gastric cancer, where this upregulation correlated with increased resistance to cisplatin (X. P. Sun, et al., 2014). These studies suggest that HIF-induced expression of IAP proteins could confer resistance to hypoxia-

induced apoptosis, simultaneously conferring on them resistance to treatment-induced apoptosis.

2.6 Metabolic Reprogramming

In order to sustain cell proliferation, tumor cells need to alter their metabolism and this has now been considered to be an emerging hallmark of cancer. It has long been known that cancer cells reprogram their metabolism by switching to glycolysis for energy production even in the presence of oxygen, and this phenomenon is called the Warburg effect. In neuroblastoma, a type of brain cancer that is predominant in children and frequently shows amplification of the Survivin gene through gain of chromosome 17q. Hagenbuchner *et al.* have shown that Survivin overexpression results in reprogramming of cancer metabolism and causes cells to shift to glycolysis from oxidative phosphorylation (OXPHOS) for energy production. Survivin mediates these effects by altering the mitochondrial fission and fusion machinery, resulting in reorganization of the mitochondrial network (Hagenbuchner, Kuznetsov, Obexer, & Ausserlechner, 2013). The same group has also demonstrated that the Survivin-induced shift to glycolysis in aerobic conditions can be therapeutically exploited using glycolysis inhibitors. The treatment of Survivin overexpressing cells with 2-deoxy-D-glucose (2DG), which is a glucose analogue and acts by inhibiting the production of glucose-6-phosphate from glucose, resulted in autophagy-mediated degradation of Survivin and re-fusion of the mitochondrial networks (Hagenbuchner, Kiechl-Kohlendorfer, Obexer, & Ausserlechner, 2016). Rivadeneira *et al.* reported that Survivin also plays a role in mitochondrial OXPHOS in prostate adenocarcinoma cells. They found that Survivin cooperates with Hsp90 to stabilize the OXPHOS complex. siRNA-mediated silencing of Survivin resulted in defective metabolic bioenergetics, increased OXPHOS

metabolites and reduced O₂ consumption (Rivadeneira, et al., 2015). These studies show that Survivin can influence both glycolysis and OXPHOS.

2.7 Immune Evasion

Another of the most striking features of tumor cells is their ability to resist eradication by the host immune system which enables cancer progression and survival of micro-metastatic cells in the circulation leading to disease dissemination. As discussed in previous sections, IAPs play an important role in regulating the NFκB pathway, a central regulator of inflammation and immunity. In response to infectious pathogen-associated molecular patterns (PAMPs) and sterile damage-associated molecular patterns (DAMPs), NFκB activation is initiated by activation of pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs), nod-like receptor (NLR) family and RIG-like receptor family (RLR), resulting in the transcription of various pro-inflammatory cytokines, chemokines and interferons. IAPs regulate activation of PRRs through their interaction with TRAF-2 and -3 and their E3-ligase ubiquitylation activity (Beug, Cheung, LaCasse, & Korneluk, 2012; Estornes & Bertrand, 2015). Additionally, IAPs regulate the adaptive immune response, functioning as negative regulators of B lymphocytes via their role in the non-canonical NFκB pathway, with cIAP depletion reported to augment B cell proliferation and survival (Zarnegar, et al., 2008).

The IAP family member Livin (ML-IAP) is expressed as two isoforms known as Livin α and Livin β, both of which have unique roles in regulating apoptosis and immunity. Livin can also be cleaved by caspases to produce a truncated form (tLivin), which has a paradoxical pro-apoptotic function. Interestingly, the Livin β isoform has been reported to protect cells against natural killer (NK) cell-mediated killing when not cleaved. However, NK cells can themselves induce cleavage of Livin to

the pro-apoptotic tLivin which can then inhibit tumor growth (Abd-Elrahman, et al., 2009; Nachmias, et al., 2007).

3. Targeting IAP proteins for cancer therapy

Due to the elevated expression of IAP proteins in many different cancers and their diverse role in tumorigenesis, they have long been considered attractive therapeutic targets. Although antisense approaches have been explored, the leading current strategies to target IAPs involve agents that mimic the interaction of the endogenous IAP antagonist SMAC with IAPs. The development of SMAC mimetic compounds, combinatorial therapies with these compounds and on-going clinical trials have been recently reviewed (Cong, et al., 2019). We summarize some of the key points below.

SMAC mimetics

The endogenous IAP inhibitor SMAC was discovered after several studies reported the presence of pro-apoptotic proteins Reaper, Head Involution Defective (HID) and Grim in *Drosophila* that could directly interact with the IAP proteins and block their caspase inhibitory activity. This led to the discovery of their mammalian counterparts. Along with cytochrome c and dATP, SMAC was discovered in mitochondrial extracts which could selectively enhance caspase-9 activity by neutralizing the effects of IAP proteins. It was also discovered in co-immunoprecipitation studies looking for binding partners of XIAP and was found to also interact with cIAP1/2. SMAC was also shown to be released into the cytosol from mitochondria during apoptosis (Du, Fang, Li, Li, & Wang, 2000; Verhagen, et al., 2000).

Structural analyses revealed that the N terminus of SMAC contains a 55 residue mitochondrial targeting sequence that is cleaved to expose a sequence of four hydrophobic amino acids (AVPI) that are required for binding to the IAP proteins. This tetra-peptide sequence binds to a conserved surface groove on the BIR3 domain of XIAP, and this sequence must be exposed for the binding to occur (Z. Liu, et al., 2000; Shi, 2002). The knowledge that SMAC binding to IAP requires only four amino acids and also the precise structural understanding of this interaction stimulated interest in development of small molecule inhibitors that could bind to this pocket. SMAC peptides derived from this sequence were shown to bind to IAP proteins and abrogate their activity and also sensitize cancer cells to chemotherapeutic agents (Franklin, et al., 2003; Vucic, et al., 2002). Furthermore, *in vivo* studies demonstrated that SMAC mimetic peptides could enhance TRAIL-induced apoptosis in xenograft models (Simone Fulda, Wick, Weller, & Debatin, 2002). These studies were the first to prove that mimicking the N-terminus of SMAC could be an effective way to target IAPs and paved the way for the development of complex fusion and other synthetic peptides (A Elsayy, G Tikhonova, Martin, & Walker, 2015; Elsayy, Martin, Tikhonova, & Walker, 2013).

However, these SMAC-based peptides did not possess the pharmacological properties to be clinically developed. Therefore, the next development was generation of small molecule compounds that mimic the 4 amino acid AVPI IAP-binding motif. This was achieved by the introduction of non-natural amino acids and various chemical substitutions in order to increase cell permeability and potency (Kipp, et al., 2002; Oost, et al., 2004). The first monovalent IAP antagonist developed by Sun *et al.* was a non-peptidic SMAC mimetic which was conformationally constrained and was 23 times more potent than the natural SMAC peptide (H. Sun, Nikolovska-

Coleska, Yang, Xu, Liu, et al., 2004). Several other SMAC mimetic compounds have now been developed that induce apoptosis in cancer cells (Cohen, et al., 2009; H. Sun, Nikolovska-Coleska, Yang, Xu, Tomita, et al., 2004; B. Zhang, et al., 2008; Zobel, et al., 2006).

Most of these monovalent IAP antagonists bind to the BIR3 domain of IAPs; however, as mentioned earlier, the BIR2 domain also plays an important role in caspase inhibition. Therefore, a third generation of IAP antagonists known as bivalent mimetics were developed consisting of two of the IAP binding tetra peptide motifs joined by a linker enabling them to simultaneously bind both the BIR2 and BIR3 domains of XIAP. One such bivalent SMAC mimetic (SM-164) was found to be several orders of magnitude more potent than its monovalent counterpart and was found to induce apoptosis at concentrations as low as 1nM in leukemia cell lines (H. Sun, et al., 2007).

The same group also looked at the ability of SMAC mimetic small molecule compounds to inhibit cIAP1/2. They investigated the potential of two different SMAC mimetics, monovalent SM-122 and bivalent SM-146 and reported that removal of cIAP1/2 alone is not enough for TNF α induced apoptosis and that XIAP plays an important role in this process (J. Lu, et al., 2008). The recently developed AZD5582 was shown to bind to the BIR2 domains of XIAP and the BIR3 domains of cIAP1/2 and XIAP. It also resulted in degradation of cIAP1 and induced apoptosis in breast cancer cell lines (Hennessy, et al., 2013). Several other bivalent SMAC mimetics have now been developed by different chemical modifications between the linker regions and were reported to be highly potent (Zide Chen, et al., 2018; W. Li, et al., 2011; Yuefeng Peng, et al., 2011; Perez, et al., 2015; Sheng, et al., 2013).

Because of their varied roles in regulating both the innate and adaptive immune system responses, IAPs are now being considered as targets for developing immunotherapy-based treatments in different cancers. In metastatic melanoma patients treated with anti-tumor vaccines, Livin was reported to stimulate a cellular and humoral immune response and is therefore a potential target for promoting immune-mediated tumor destruction (J. Schmollinger & Dranoff, 2004; J. C. Schmollinger, et al., 2003). Similar reports have also been published for Survivin with it being capable of inducing tumor-specific CD8⁺ and CD4⁺ T cells in colorectal cancer and cytotoxic T cells in chronic lymphatic leukemia, breast cancer and melanoma (Andersen, Pedersen, Becker, & Thor Straten, 2001; Andersen, Pedersen, Capeller, et al., 2001; Casati, et al., 2003). Furthermore, various studies have shown that IAP antagonists have co-stimulatory effects on T cells and can enhance the potency of tumor vaccines (Dougan, et al., 2010). Importantly, these agents have been reported to synergize with immune checkpoint inhibitors: Beug *et al.* were the first to report synergism between IAP antagonists and immune checkpoint inhibitors in a mouse model of glioblastoma in which monotherapy was ineffective. They reported that combined treatment with LCL161, which is an orally bioavailable IAP inhibitor targeting XIAP and cIAP1/2, and anti-PD1/CTLA4 antibodies significantly increased survival rates of the mice. This synergy was reported to be dependent upon TNF α inducing cytotoxic T cell activity and also type I interferon signaling (Beug, et al., 2017). Similar findings were reported in mouse models of multiple myeloma, where the median survival of the mice co-treated with LCL161 and anti-PD1 antibody increased to 33 days from 14 days for the treatment with the anti-PD1 antibody alone; moreover, this combination was curative in all mice that completed this combinatory treatment for 2 weeks (Chesi, et al., 2016). Since PD1 expression diminishes production of TNF α and IAP antagonists sensitize tumor cells to cytotoxic T cell-derived TNF α ,

combining these two strategies can enhance T cell-mediated killing of tumor cells (Kearney, et al., 2017).

The advancements in the development of IAP antagonists along with the increased understanding of IAP protein biology has paved the way for the use of IAP antagonists in clinical trials. Both monovalent and bivalent SMAC mimetics have entered clinical trials and are being tested to determine their pharmacological, dosing, and safety characteristics. Additionally, a non-peptidomimetic orally administered IAP antagonist (ASTX-660) has also been developed by fragment-based design and is currently in early-phase clinical trials (Derakhshan, et., 2017). The list of IAP antagonists in Phase II clinical trials is summarized in Table 2.

3.2 Biomarkers for treatment response to SMAC mimetics

For the effective implementation of SMAC based therapies in the clinic, identification of biomarkers to identify patients most likely to benefit from treatment with them are critical. It has been reported that the anti-tumor effects of SMs depends upon the ability of tumors to produce and respond to TNF α or any TNF α -associated death ligand, and tumors that are unable to carry out either of these functions are most likely to be resistant to SM based therapies (Gaither, et al., 2007; Petersen, et al., 2007). Preclinical studies in breast cancer xenograft models assessing potential biomarkers of treatment response suggested that plasma levels of TNF α and cleaved and total cytokeratin-18 (a marker of caspase-dependent cell death) may be useful (Sumi, et al., 2013). However, in a clinical trial of Debio 1143 in patients with advanced cancer, plasma levels of TNF α and cytokeratin-18 along with other inflammatory markers like IL-8 and CCL2 showed no apparent relationship with treatment response. (Hurwitz, et al., 2015).

In a recent study, Zinngrebe *et al.* reported the identification of gene expression markers that could help in stratifying patients that were particularly sensitive to SMs. This was done in B-cell acute lymphoblastic leukemia (BCP-ALL) and found four commonly predictive genes, which were *TSPAN7*, *DIPK1C*, *MTX2* and *TNFRSF1A*, the latter encoding TNFR1 (Zinngrebe, et al., 2020). Additionally, it was recently reported that systems biology approaches to mathematically model apoptosis competency may be useful for predicting response to IAP antagonists; these algorithms correlated with efficacy in CRC xenograft models and indicated that resistance to IAP antagonists can be overcome by combining with standard-of-care chemotherapy (Crawford, et al., 2018).

These studies highlight the need for the development of predictive biomarkers for IAP antagonists that could be used to identify patients that would benefit from these treatments and also whether these therapies could be added to standard-of-care chemotherapies.

4. Conclusion

As outlined above, there is extensive literature linking IAPs to different hallmarks of tumorigenesis: sustained proliferation, invasion and metastasis, angiogenesis, evasion of cell death, evasion of anti-tumor immunity and tumor-promoting alterations in metabolism. While their role in regulating apoptosis is well studied, a deeper understanding of their involvement in other cellular processes is needed in order to fully exploit therapeutic targeting of IAPs. Overexpression of IAPs in a number of cancers makes it an attractive target, and there have been a number of agents developed which are showing promise in clinical trials. For clinical approval, it will be important to identify predictive biomarkers to enable selection of the target patient population. While signals of single agent activity have been observed, it is likely that anti-IAP therapies have their greatest potential in combination with other agents, including standard-of-care chemotherapeutic agents, targeted agents and radiotherapy.

Acute Myeloid Leukemia						
Biospecimen analyzed	Number of patients	IAP protein studied	Expression	mRNA/protein level	Prognosis/Comments	Reference
Bone marrow aspirates	120	clAP2	High	mRNA	Poor OS (part of a 3 gene signature)	Hess, et al., 2007
Blood	78	XIAP	Low	Protein	Better OS & Remission duration	Tamm, et al., 2000
Blood & bone marrow	62	XIAP Survivin, clAP1	Low -	Protein Protein	Better OS No prognostic impact	Tamm, et al., 2004
Blood	172	XIAP, Survivin	-	Protein	No prognostic impact	Carter, et al., 2003
Blood & bone marrow	56	SMAC/Diablo Survivin XIAP, clAP1, clAP2	High Low -	Protein Protein Protein	Better OS, No impact on DFS Better OS, No impact on DFS No impact on OS & DFS	Pluta, et al., 2015
Bladder Cancer						
Tissue	176	XIAP	High	Protein/mRNA	Lower RFS, Independent prognostic factor for recurrence	Ming, Tao, Yin, & Na, 2007
Tissue	102	clAP1, clAP2	High (nuclear)	Protein	Poor OS & RFS	X. Che, et al., 2012
Tissue	105	XIAP, clAP1, clAP2, Survivin	High	Protein	Reduced RFS	X. Chen, at al., 2013
Tissue/Urine	-	Survivin	High	Protein/mRNA	Poor prognoses predictor for OS, RFS and PFS (Systematic Review)	Jeon, Kim, Kwak, Kim, & Ku, 2013
Breast Cancer						
FFPE Tissue	102	XIAP, Smac	High (nuclear)	Protein	Poor OS	Y. Zhang, et al., 2011
Tissue	293	Survivin	High (nuclear)	Protein	Independent predictor of OS & RFS	Kennedy, et al., 2003
Tissue	92	XIAP, clAP1, clAP2, survivin	Low	Protein	Longer PFS, No impact on OS	P. Pluta, et al., 2015
Pancreatic Cancer						

Cell lines	-	clAP2	High	mRNA	Resistance to chemotherapeutic drugs	Lopes, Gangeswaran, McNeish, Wang, & Lemoine, 2007
Tissue	33	clAP1, clAP2	High	Protein/mRNA	Co-expression predicts worse prognosis, clAP2 levels increase during disease progression	Esposito, et al., 2007
Tissue	89	XIAP associated factor (XAF1)	Low	Protein	Shorter OS, Independent predictor of survival	J. Huang, et al., 2010
Renal Cell Carcinoma						
Tissue	109	XIAP	High	Protein	Correlates with tumour stage, shorter DFS	Mizutani, et al., 2007
FFPE Tissue	145	XIAP	High	Protein	Correlates with tumour stage, postoperative survival	Ramp, et al.,2004
Non-small cell Lung Cancer						
FFPE tissue (advanced stage tumours)	55	XIAP, clAP1, clAP2	-	Protein	No impact on OS & PFS, response to chemotherapy	Ferreira et al., 2001
FFPE tissue (early stage tumours)	144	XIAP	High	Protein	Longer OS	Ferreira et al., 2001
Colorectal Cancer						
Tissue	96	XIAP	High	mRNA/Protein	Correlates with tumour differentiation, stage and invasion, DFS & OS	Xiang, Wen, Wang, Chen, & Liu, 2009
Tissue	106	clAP2	High	Protein	Shorter OS	Krajewska, et al., 2005
Cervical Cancer						
Cell lines/Tissues	70	clAP1	High (nuclear)	Protein	Correlation with resistance to radiation induced cell death, reduced OS & PFS	Imoto, et al., 2002
MALT Lymphoma						
Cell line	-	clAP2	-	Protein	Fusion protein with MALT associated with advanced stage disease	Rosebeck, et al., 2011
Hepatocellular carcinoma						
Tissue	80	XIAP, XAF1	High	mRNA	Shorter OS, XIAP to XAF1 ratio correlates with reduced OS	Augello, et al., 2009
Gastric adenocarcinoma						
Tissue	46	XIAP, XAF1	High	mRNA	XIAP to XAF1 ratio correlated with reduced OS	Shibata, et al., 2007

Table 1: IAP expression and correlation to prognosis

Agent	Condition	Combination	Number enrolled	NCT / EudraCT Number	Study Start	Study Completion	Status	Conclusions/Findings	Reference
Debio 1143	Squamous Cell Carcinoma of the Head and Neck	Cisplatin + Radiotherapy	110	NCT02022098 / 2013-000044-25	Oct-13	Apr-20	Active, not recruiting	Improved locoregional control and PFS, good tolerability	Bourhis et al., 2019
	Solid Tumors	Nivolumab	72	NCT04122625	Apr-19	Jan-23	Recruiting	-	-
	Squamous Cell Carcinoma of the Head and Neck	Cisplatin	24	2014-004655-31	Aug-15	Jul-18	Completed	Good distribution into tumours, targeted cIAP1, increased CD8+ lymphocytes and PD-1/PD-L1 positive immune cells	Gomez-Roca et al., 2019
	Solid Tumors	Nivolumab	69	2018-003546-16	May-19	Ongoing	Recruiting	-	-
	Epithelial Ovarian	Carbolplatin + Paclitaxel	35	2015-005137-42	Jun-16	Jan-18	Terminated early	Underdosing of paclitaxel in combination arm revealed by PK analysis	
LCL161	Recurrent or refractory Plasma Cell Myeloma	Cyclophosphamide	25	NCT01955434	Nov-13	Jun-17	Completed	-	-
	Polycythemia Vera, Post-Polycythemic Myelofibrosis Phase, Primary Myelofibrosis, Secondary Myelofibrosis	Monotherapy	40	NCT02098161	Dec-14	Dec-21	Recruiting	Reported a 30% response rate in treatment resistant population	Pemmaraju et al., 2018
	Small Cell Lung Cancer, Ovarian Cancer	Topotecan	52	NCT02649673	Mar-16	Jul-19	Recruiting	-	-
	Triple Negative Breast Cancer	Paclitaxel	209	2012-000677-23	Aug-12	Sep-14	Completed	Addition of LCL161 did not improve pathological CR, TNFα gene signature associated with higher response rates	Bardia et al., 2018; Parton et al., 2015

ASTX660	Solid Tumors	Monotherapy	230	NCT02503423	Jul-15	Oct-21	Recruiting	Preliminary results suggest that it shows activity in a T cell lymphoma cohort, manageable safety profile	Mehta, et al., 2019
	Relapsed or Refractory Peripheral T-cell Lymphoma (PTCL), Cutaneous T-cell Lymphoma (CTCL), Adult T-cell Leukemia/Lymphoma (ATLL)	Monotherapy	61	NCT04362007	Apr-20	Sept-24	Recruiting		
Birinapant	Solid Tumors	Pembrolizumab	135	NCT02587962	Aug-17	Jun-21	Terminated	Lack of clinical benefit, Preliminary results suggest that the combination with pembrolizumab is safe and tolerable	Schilder et al., 2019
	Myelodysplastic Syndrome	5-Azacitidine	21	NCT01828346	Jun-13	Nov-15	Completed No results		
	Myelodysplastic Syndrome (MDS), Chronic Myelomonocytic Leukemia (CMML)	Azacitidine (AZA) or placebo	118	NCT02147873	Jun-14	Jun-16	Terminated early	Response rate of combination not superior to AZA alone, serious and adverse events rate higher in birinapant arm	Donnellan, et al., 2016
	Solid Tumors	Carboplatin/Paclitaxel, Irinotecan, Docetaxel, Gemcitabine, liposomal doxorubicin (multi-arm)	176	NCT01188499	Oct-10	Mar-14	Completed	Combination with irinotecan demonstrated clinical benefit in irinotecan relapsed/refractory patients with colorectal cancer	Senzer et al., 2013
	Epithelial Ovarian Cancer, Peritoneal Neoplasms or Fallopian Tube Neoplasms	Monotherapy	11	NCT01681368	Aug-12	Apr-14	Terminated early	Lack of clinical benefit after treatment with birinapant	Noonan et al., 2016
	Acute Myelogenous Leukemia	Monotherapy	7	NCT01486784	Nov-11	Ongoing	Terminated	-	-

APG-1387	Advanced Solid Tumors	Toripalimab	108	NCT04284488	Apr-20	Apr-24	Not yet recruiting	-	-
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Table 2: IAP antagonists in Phase II clinical trials

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