Introduction

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Review of Literature

1.0 Introduction

Cancer cells have characteristics to acquire a number of cellular physiological alterations during the course of tumor progression. These include the capabilities of independent proliferative potential, insensitivity to anti-growth signals, sustained angiogenesis, loss of cell cycle control, promotion of tumor microenvironment to invade surrounding tissues, metastases to distant sites and evasion of programmed cell death mechanism to make them malignant (Hanahan and Weinberg, 2000). These characteristics differentiate normal versus cancerous cell that reflects alterations in the cellular signaling pathways responsible for the fate of cell death or survival. Programmed cell death (Apoptosis) has been implicated as a physiological cell death, which is an essential to maintain cellular homeostasis in multi-cellular organisms. Dysregulation of apoptosis may lead to various diseases in humans, such as cancer, AIDS, neurodegenerative, autoimmune diseases, inflammatory and infectious disorders (Grivennikov et al., 2010; Grivennikov and Karin, 2010). Apoptosis is mainly triggered by activation of caspases through complex signaling pathways, which includes death receptor (extrinsic) and mitochondria dependent (intrinsic) signaling (Thornberry and Lazebnik, 1998; Scaffidi et al., 1998; Wang, 2001; Dempsey et al., 2003; Kim et al., 2009; Tait and Green, 2010). The death receptor mediated apoptosis is mainly contributed by group of receptors like CD95, TRAIL-R1 and TRAIL-R2 belongs to the tumor necrosis factor-1 (TNF-1) receptor superfamily-1 and transduces extrinsic signaling of programmed cell death (Ashkenazi and Dixit, 1998; Choi and Benveniste, 2004; Elmore, 2007; Guicciardi and Gores, 2009). In contrast, activation of death receptor signaling also coordinates with mitochondrial mediated apoptosis followed by activation of initiator and executioner caspases (Werner et al., 2002; Elmore, 2007; Czabotar et al., 2014). The activation of death receptors has a complex signaling network to transduce programmed cell death by apoptosis. These receptors have a characteristic death domain (DD) at their cytoplasmic tail, which homophilically interacts with DD containing adaptor protein Fas-associated death domain (FADD) for transducing downstream apoptotic signaling (Chinnaiyan et al., 1995; Dempsey et al., 2003; Tourneur and Chiocchia, 2010). FADD contains two distinct domains, C-terminal death domain (DD) and N-terminal death effector domain (DED) which provide docking site for homophilic interaction, oligomerization and autocatalytic processing to activation of downstream initiator caspases. The DED of FADD allows to recruit DEDs carrying proteins like pro-caspase-8/10, which in turn initiates the formation of death inducing signaling complex (DISC) (Tourneur et al., 2005; Scott et al., 2009; Tourneur and Chiocchia, 2010). The

initiation of DISC formation facilitates autocatalytic processing of procaspases 8 /10 to activate downstream executioner caspases such as caspase-3 and caspase-7 for the commencement of apoptotic cell death (Li et al., 1998; Kim et al., 2009; Peter and Krammer, 2003). The death receptor mediated apoptosis is negatively regulated by anti-apoptotic protein cFLIP (Chang et al., 2002; Jonsson et al., 2003; Golks et al., 2005; Piao et al., 2012; Safa, 2012). Several reports highlight that FADD and cFLIP are associated with apoptotic and non-apoptotic functions including cell proliferation, cell cycle progression, tumor development, inflammation, innate immunity and autophagy (Chinnaiyan et al., 1995; Irmler et al., 1997; Krueger et al., 2001). Previous reports demonstrate that low expression of FADD and elevated expression of cFLIP are associated with progression of malignancy (Golks et al., 2005; Tourneur and Chiocchia, 2010; Safa, 2012). Thus, FADD and cFLIP play an important role in the regulation of cell death and survival pathways. However, the cellular signaling of FADD and cFLIP to determine the fate of cell death or survival remains to be elucidated.

Interestingly, the fate of cell death or survival also depends upon secretion of endogenous stimulant. The cytokine TNF-α is a pleotropic endogenous molecule that plays dual role in cell death and survival (Wajant et al., 2003). Activation of cellular signaling by TNF-α and its receptor transduces multiple cellular responses ranging from inflammation to apoptosis and other forms of cell death (Leong and Karsan, 2000; Muppidi et al., 2004). TNF Receptor (TNFR) signaling elicits non-apoptotic or apoptotic response by formation of two sequential complexes depending upon the stimulation of the ligand. For instance, the binding of TNF-α to TNFR induces formation of complex I with the association of TRADD, TRAF2 and cIAP1/2 to ubiquitinate RIP1 for the activation of NF-κB and associate gene product cFLIP (Feoktistova et al., 2011; Brenner et al., 2015). Subsequently, the ablation of cIAPs ubiquitin activity dissociates RIP1 from complex I to form complex II accompanied with FADD and caspase-8 for the execution of apoptotic cell death (Micheau and Tschopp, 2003; Wang et al., 2008). Surprisingly, $cFLIP_L$ also provides stability to TNFR1 signaling complex I for constitutive activation of NF-κB and cell proliferation (Kreuz et al., 2001; Micheau et al., 2001). However, involvement of FADD in regulation of NF-κB and cFLIP is not fully understood.

In fact, cellular homeostasis in multi-cellular organism is maintained by distinct modalities of cell death, including apoptosis, necrosis and autophagy (Nikoletopoulou et al., 2013). However, the different forms of cell death are evolutionarily conserved processes to regulate the cell fate. Apoptosis and autophagy both are sequential processes

to remove damaged or unwanted cellular content (Thorburn, 2008). Consequently, a defect in apoptosis and autophagy has been associated with various diseases, including neurodegenerative disorder, diabetes, aging, infection, autoimmunity and cancer (Levine and Kroemer, 2008; Livesey et al., 2009; Choi et al., 2013). The process of autophagy generally referred as "self-eating" process that tends to degrade the unwanted cytosolic constituents by transporting them to lysosome. Although, basal level of autophagy helps to maintain homeostasis in normal conditions, while during stress conditions autophagy also supports cell survival (Bergamini et al., 2004; Levine and Klionsky, 2004). Recent reports suggest that cell death regulatory proteins, including FADD and cFLIP regulate apoptosis and autophagy (Pyo et al., 2005; Pua et al., 2007; Lee et al., 2009). The DD of FADD interacts with autophagic protein Atg5 and commences autophagic cell death (Thorburn et al., 2005; Bell et al., 2008). Recent reports state that cFLIP suppresses autophagy by inhibiting the interaction of Atg3 with LC3 and represses cell death with autophagy (Lee et al., 2009; Young et al., 2012). Taken together, both the processes autophagy and apoptosis require tight control over their constituent proteins to decide the fate of cells. Moreover, NF-κB induced cFLIP activation is ubiquitously primed by pro-inflammatory cytokine TNF-α (Micheau et al., 2001). Previously, it has been shown that TNF-α induced activation of NF-κB represses autophagy, however ablation of NF-κB triggers autophagic cell death (Djavaheri-Mergny et al., 2006). The elusive role of TNF-α and cFLIP in cell survival by autophagy pathway is poorly understood. Therefore, unraveling the cross-talk between FADD and cFLIP in regulation of apoptosis and autophagy may explore to define cellular fate for cell death and survival.

Recent report reveals that inflammation is also a major hallmark of the cancer (Hanahan and Wienberg, 2011). Inflammation creates a complex microenvironment of pro-inflammatory mediators that promotes neoplastic transformation of cells (Grivennikov and Karin, 2010). The activated immune cells, such as macrophages, dendritic cells and endothelial cells, which release numerous inflammatory mediators, such as cytokines, chemokines, leukotrienes prostaglandins (PGs), reactive oxygen species (ROS) and nitric oxide (NO), which helps in tumor promotion by altering normal cellular signaling cascades (Coussens and Werb, 2002; Grivennikov et al., 2010). Such inflammatory mediators are regulated by nuclear transcription factors NF-κB and play major role in tumorigenesis (Karin and Greten, 2005). A recent report suggests that Inflammatory mediators and inhibition of apoptosis are a major giver for cell survival and malignancy (Wallach and Kovalenko, 2014). Inflammation is a complex biological response to harmful stimuli, which immediately activates the immunological defence

system and protects the tissue from damage. Therefore, inflammation is essentially beneficial at earlier stage but, excess or prolonged inflammation promotes tumor microenvironment for malignancy (Kepp et al., 2009; Grivennikov and Karin, 2010). A link between inflammation and cancer has long been proposed, but its molecular signature and cross talk between cell death and survival remains to be elucidated. In contrast, the dying tumor cells may release inflammatory cytokines such as IL-1β and IL-18 by activating NLRP3 inflammasome to suppress the apoptotic signaling (Abderrazak et al., 2015). Consistent with this, FADD has also been linked in the regulation of inflammatory activation apart from its role in apoptosis, necroptosis and autophagic signaling (Hsu et al., 1995; Ponton et al., 1996; Rensing-Ehl et al., 1995; Desbarats et al., 2000; Desbarats et al., 2003). However, involvement of FADD in regulation of inflammasome mediated activation of IL-1β is not well explored.

Thus, FADD is an important regulator for determining the fate of cell death or survival and it could be a promising candidate to offer a novel strategy for cancer therapy. In the present study, cellular dynamics of FADD in regulation of cell death, survival and inflammatory signaling was explored.

1. 1 Objectives

These following objectives were proposed to achieve the aim of the present study:

1. *To study the apoptotic potential of FADD by over expression and explore the expression of pro and anti-apoptotic proteins involved in apoptotic signaling.*
2. *To explore the mechanism how FADD regulates expression of cFLIP and activation of nuclear transcription factor NF-kB at the cellular level.*
3. *To study the role of FADD on the regulation of autophagy (survival) pathway.*
4. *To explore the mechanism of cFLIP mediated activation of nuclear transcription factor NF-kB and inflammatory cytokines in cancer cells.*
5. *To carry out cloning of human FADD.*
6. *To carry out protein purification and characterization of Human FADD.*
7. *Synthesis and characterization of FADD conjugated cell permeable peptides (CPP).*
8. *Examine the delivery of CPP-FADD inside the cells.*
9. *To study the apoptotic and inflammatory response of cell permeable peptides conjugated FADD (CPP-FADD) delivery inside the cells.*
10. *To study the comparative effects of CPP-FADD and molecule responsible for activation apoptotic cell death.*

1.2 Review of Literature

1.2.1 Cancer and Apoptosis

Cancer is a hyper proliferative disorder in which cancer cells possess a great ability to mutate, rapidly grow and escape from the programed mechanism of cell death (Hanahan and Weinberg, 2000). The process of cell death can be classified according to the nature of the stimuli or the involvement of catastrophic components of cell death. In fact, cellular homeostasis in multi-cellular organism is maintained by distinct modalities of cell death including apoptosis, autophagy and necroptosis as shown in Figure 1. Mechanistically, apoptosis and autophagic cell death are generally considered as immunologically silent processes; however, necrotic death may induce a local inflammatory response as a cellular defense mechanism (Nikoletopoulou et al., 2013). The apoptosis is mainly triggered by activation of death receptors (extrinsic) and change in mitochondrial integrity (intrinsic) (Taylor et al., 2008; Tait and Green, 2010). Death receptors (DRs) belong to the TNF receptor (TNFR) superfamily, such as Fas (CD95/ Apo1) and TNFR1 (p55/CD120a), contain a conserved module of approximately 80–100 amino acids long cytoplasmic death domain (DD) at its cytoplasmic tail, which homophilically interacts with cytosolic DD containing pivotal adaptor proteins such as Fas-associated death domain (FADD/MORT1) or TNFR-associated death domain (TRADD) adaptor protein to regulate diverse mechanisms of cell signaling, like apoptosis, proliferation and differentiation (Bender et al., 2005; Tourneur et al., 2005). In contrast, the FADD contains two distinct domains, C-terminal death domain (DD) and N-terminal death effector domain (DED), which provides docking site for homophilic interaction, oligomerization and autocatalytic processing to activation of downstream apoptotic signals (Chinnaiyan et al., 1995; Scott et al., 2009). The DD of FADD interacts with DD of the death receptors and DED allows to recruit DEDs carrying proteins like pro-caspase-8/ 10, to initiate the formation of a death inducing signaling complex (DISC) (Holler et al., 2003). The DISC facilitates autocatalytic processing and activation of procaspases 8/10 and the release of active enzyme into the cytoplasm to cleave and activate effector caspases such as caspase-3 and caspase-7, leading to the activation of apoptotic cascades (Figure 2) (Chinnaiyan et al., 1995; Peter and Krammer, 2003). However, a defect in the process of apoptosis machinery leads to rapid cell proliferation in tumors by altering the expression of apoptosis regulatory proteins (Pommier et al., 2004). It is well documented that the apoptotic cell death signaling is regulated by anti-apoptotic proteins cIAPs, XIAP, some of Bcl-2 family members and cFLIP (Adams and Cory, 2007; Czabotar et al., 2014). Dysregulation of apoptosis is a major factor for progression of malignancy and it has been considered as a hallmark of cancer

(Hanahan and Weinberg, 2000). Recent advances in cancer therapeutics suggest that manipulation of apoptosis may be applicable for cancer therapy (Call et al., 2008). Since last decades, major attention has been given to induction of apoptosis by using small molecule as anti-cancer agents. In contrast, apoptosis can also be induced by modulating the intrinsic signaling components of the cell, which could open a new avenue for cancer therapy.

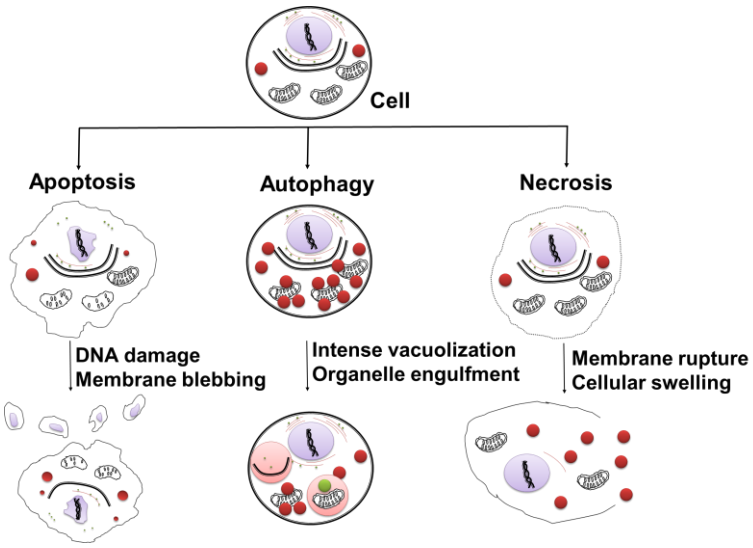

Figure 2. Modalities of Cell Death. Death pathways can follow different subroutines; the most prominent mechanism are: Apoptosis- the programmed cell death (type 1 cell death), autophagic (type 2 cell death) and necrosis - massive swelling of cytoplasmic organel) and necrosis - massive swelling of cytoplasmic organelles (immunologically harmful).

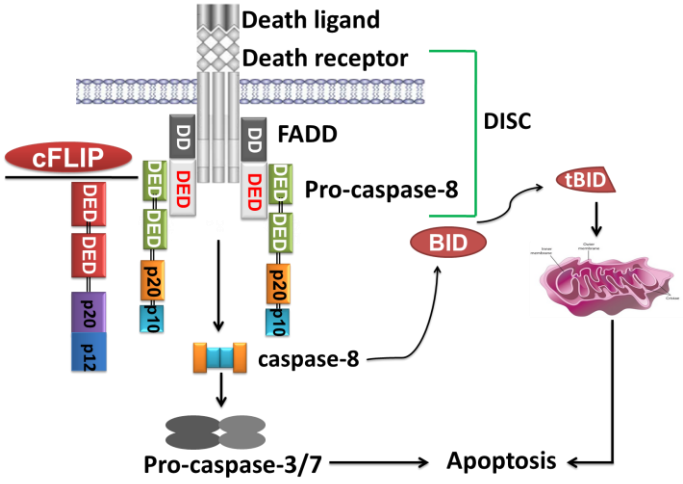

Figure 1. Death receptor apoptosis signaling. Binding of Fas ligand to Fas receptor (FasR) induces trimerization of the receptor followed by recruitment of FADD, procaspase-8. The complex of FasR-FADD-procspaese-8 forms death inducing signaling complex (DISC) which facilitates processing and activation of procaspase-8. The activated caspase-8 leads to induction of extrinsic and intrinsic (mitochondrial) cascade of apoptosis. The caspases-8 inhibitor cFLIP binds to FADD and blocks caspases-8 activation.

1.2.2 cFLIP: Regulator of apoptosis and autophagy signaling

Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (cFLIP) is a major anti-apoptotic protein and plays an important role in regulation of death receptor (DR) mediated apoptosis to promote cell survival by modulating various signaling pathways (Bagnoli et al., 2009; Piao et al., 2012). An earlier report suggests that downregulation of pro-apoptotic molecules like Fas and caspase-8 or upregulation of cFLIP blocks the death receptor signaling (Yu et al., 2009). The anti-apoptotic cFLIP is a death effector domain (DED) containing protein and has structural similarities with procaspase-8 and -10, but lack cysteine residue for autocatalytic activity (Krueger et al., 2001). Notably, the gene encoding cFLIP is in close vicinity of the genes encoding caspase-8 and caspase-10 on chromosome 2q33, which may interlink the three paralogs at the genetic level (Irmler et al., 1997). The adaptor protein FADD provides a docking site for binding to both procaspase-8 and cFLIP to regulate cell death and survival (Chinnaiyan et al., 1995; Peter and Krammer, 2003). The anti-apoptotic protein cFLIP competitively inhibits the binding and activation of procaspase-8 at the DISC and hinders apoptosis when expressed at a high level as shown in Figure 3. By contrast, it was noticed that cFLIP has more binding affinity to FADD as compared to caspase-8 at the DISC (Thome et al., 1997; Scaffidi et al., 1998; Chang et al., 2002). Moreover, some interesting findings reveal that low binding of FADD or caspase-8 at the DISC abrogates the downstream signaling upon death receptor (FasR) stimulation (Fulda et al., 2001; Tourneur et al., 2003). The anti-apoptotic functions of the cFLIP are tightly governed by their expression levels (Krueger et al., 2001). Beside that the basal level of the cFLIP has shown an important role in maintaining cellular homeostasis and other non-apoptotic functions (Matsuda et al., 2008). The expression of cFLIP was found in many cell types such as endothelial cells (Imanishi et al., 2000; Bannerman et al., 2001), keratinocytes (Irisarri et al., 2000) or dendritic cells (Willems et al., 2000) and are found in abundance in the heart tissue, skeletal muscles, lymphoid tissues and kidney cells (Irmler et al., 1997; Budihardjo et al., 1999).

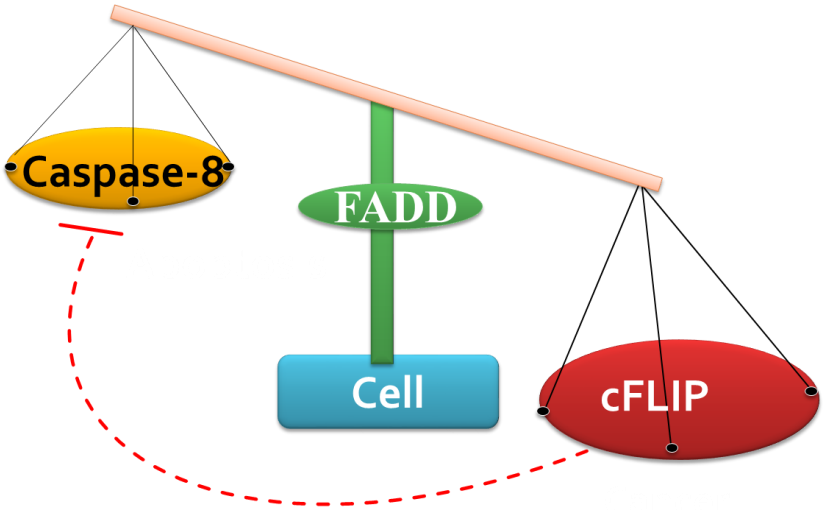

Figure 3. Binding of caspase-8 and cFLIP to FADD at DISC. The adaptor protein FADD provides common binding sites for proapoptotic pro-caspase-8 and anti-apoptotic cFLIP at DISC. The cFLIP competitively excludes the binding of procaspase-8 to FADD and inhibits apoptosis.

The cFLIP has three different isoforms such as long form $cFLIP_L$ (55 kDa), a short variant $cFLIP_S$ (27 kDa) and $cFLIP_R$ (25 kDa) (Figure 4). All the variants of cFLIP contain two death effector domains (DED), but $cFLIP_L$ has one inactive caspase like domain, whereas $cFLIP_S$ and $cFLIP_R$ lack the entire caspase like domain (Longley et al., 2006; Ozturk et al., 2012). Indeed, all the isoforms of cFLIP are believed to function as a competitive inhibitor of procaspase-8 for recruitment to the DISC *via* their DEDs when expressed at high levels, and low level of cFLIP promotes activation of caspase-8 (Bagnoli et al., 2009; Yu et al., 2009). The cFLIP proteins are very short lived and require modification at the post-translational level (Fulda et al., 2001; Kreuz et al., 2001; Micheau et al., 2001). The phosphorylation of $cFLIP_L$ and $cFLIP_S$ has been found in different origins of cell lines, which inhibits the interaction of $cFLIP_L$ and $cFLIP_S$ with the adaptor molecule FADD and sensitizing cells towards apoptosis (Higuchi et al., 2003). Furthermore, it has also been shown that the different forms of cFLIP were modified post-transnationally by ubiquitination signaling (Fukazawa et al., 2001; Zhang et al., 2004a). The expression of cFLIP has been regulated by E3 ubiquitin ligases (Chang et al., 2006). Previously it has been shown that expression of cFLIP has been up-regulated upon TNF-α induced activation of transcription factor NF-κB (Kreuz et al., 2001; Golks et al., 2006; Marques-Fernandez et al., 2013). Notably, activation of the NF-κB also induces the expression of Bcl-2 and associated cellular signaling component (Beg and Baltimore, 1996; Cleland et al., 2011; Micheau et al., 2001; Karin and Lin, 2002). The cFLIP is also known to regulate autopahy signaling (Lee et al., 2009). The cellular processes of autophagy and apoptosis are critically regulated by various sets of gene responsible for cell death and survival

(Levine and Yuan, 2005). The interaction between anti-apoptotic protein Bcl-2 and the autophagic protein Beclin-1 regulates the formation of autophagosome during autophagic stress (Pattingre et al., 2005; Maiuri et al., 2007; Oberstein et al., 2007). Recent reports advocate that during stress condition the nuclear binding protein HMGB1 translocates to the cytosol and dissociates Bcl-2 from Beclin-1 to sustain autophagy (Kang et al., 2010; Tang et al., 2010). However, the involvement of anti-apoptotic regulators such as $cFLIP_L$ and Bcl-2 in the regulation of autophagy and nuclear to cytoplasmic translocation of HMGB1 remains elusive (Figure 5). An earlier report suggests that cFLIP abrogates the conjugation of autophagic protein Atg3 to LC3 and masks autophagic induction (Lee et al., 2009). However, despite the significant advances in understanding the fate of cell death and survival, the involvement of FADD and cFLIP in regulation apoptosis and autophagy remains elusive (Figure 6).

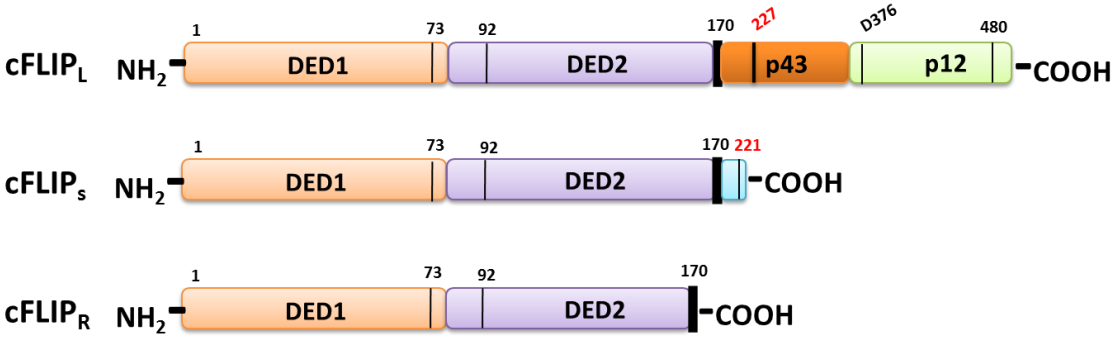

Figure 5. Different isoforms of cFLIP in Human. cFLIP has $cFLIP_L$, $cFLIP_S$ and $cFLIP_R$ isoforms. All the isoforms carries two DEDs at N-terminal but, at C-terminal the sequences varies from each other. The isoforms of cFLIP were reportedly involves in blockade of apoptosis signaling.

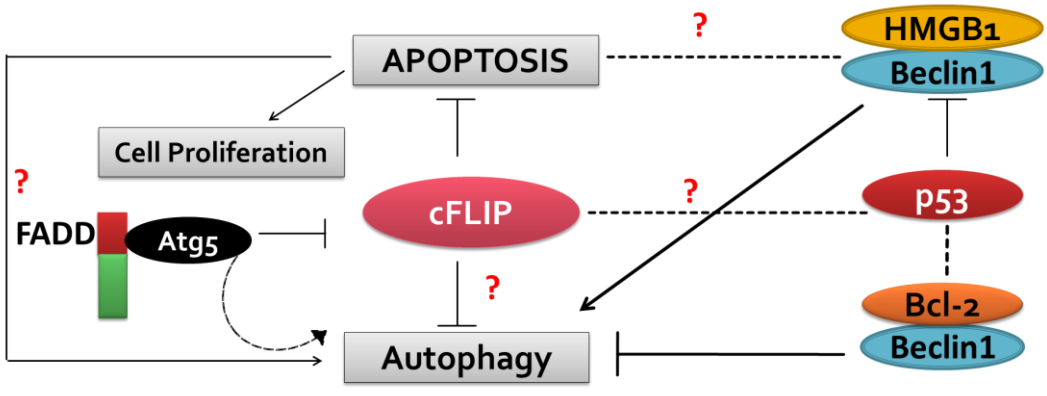

Figure 4. cFLIP in regulation of Autophagy. The canonical interaction of autophagic protein Beclin 1 with Bcl-2 suppresses autophagic stress. During cellular stress nuclear binding proteins HMGB1 and p53 modulates Beclin 1-Bcl-2 interaction to sustain autophag. The role of cFLIP in retaining the Beclin 1-Bcl2 interactions to supress autophagic stress remain poorly resolved.

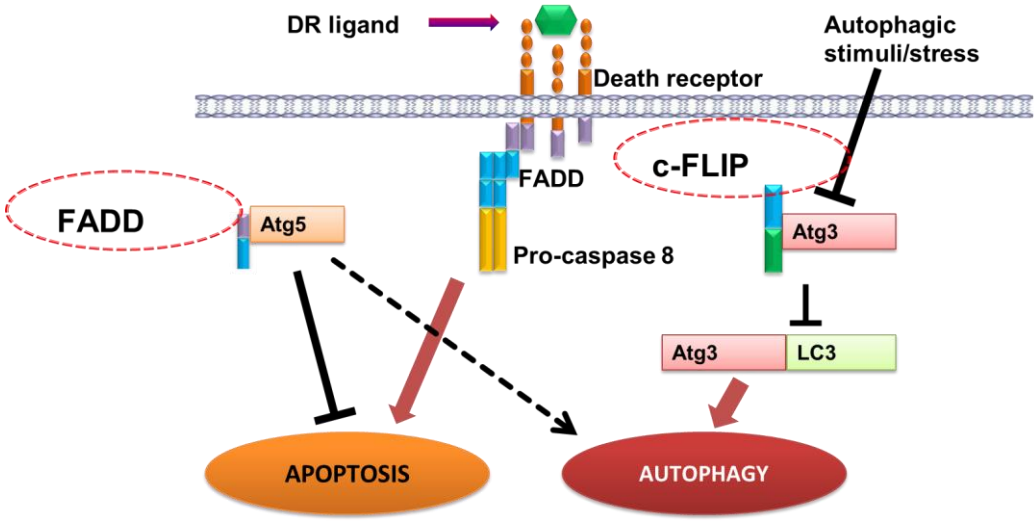

Figure 6. Involvement of FADD and cFLIP in regulation of Apoptosis and Autophagy. The process of apoptosis and autophagy are interlinked by key regulatory proteins responsible for cell death and survival. FADD interacts with Atg5 via death domain (DD)-DD to induction of autophagy during stress conditions apart from their apoptotic function. In contrast, the anti-apoptotic protein cFLIP interacts with Atg3 in order to supress autophagic stress. Interestingly, both the proteins play an important role in apoptosis and autophagy to determine fate of cell death or survival.

1.2.3 Cellular dynamics of FADD

Fas Associated Death Domain (FADD) is an adaptor protein that is known to be important for death receptor (DR) signaling of apoptosis. Besides being an essential component of apoptosis, FADD has been shown to regulate other non-apoptotic functions such as cell cycle progression, cell proliferation, embryogenesis, innate immunity, inflammation, tumorogenesis and autophagy (Beisner et al., 2003; Tourneur et al., 2005; Werner et al., 2006; Tourneur and Chiocchia, 2010). In fact, FADD is a multi-functional protein that participates in various cellular signaling pathways. Upon binding of ligand to the cell surface receptors, the DD of cell surface receptor homophilically interacts with the DD of FADD and induces oligomerization of the death effector domains (DEDs) of FADD with apical caspases such as, caspase 8/10 to form a DISC (Tourneur and Chiocchia, 2010). In the downstream, DISC facilitates processing and catalytic activation of caspases-8/10 to commence apoptotic cell death (Scaffidi et al., 1998). Since the first role ascribed to FADD was known to transduce death receptor signaling through its interaction with membrane bound death receptors and it was assumed that FADD localized in the cytoplasm, but later on it was found that the expression of FADD was also present in the nucleus (O'Reilly et al., 2004; Fricsh et al., 2004). Although, it has been shown that expression of FADD in the nucleus protects cells from apoptosis, but molecular mechanism for cell survival has not been clearly defined (Gomez-Angelats and Cidlowski, 2003). The physiological significance of FADD nuclear-cytoplasmic trafficking is based on cell and cancer type

(Screaton et al., 2003). FADD is known to constitutively express in normal cell to maintain cellular homeostasis, however defective or low expression of FADD is associated with pathological consequences associated with evasion of apoptosis and progression of cell survival by activating various signaling components (Tourneur et al., 2003). In fact, low expression of FADD was found in various types of mouse and human cancer cells and contributes to resistance to apoptosis (Tourneur and Chiocchia, 2010). An earlier report showed that, deficiency of FADD leads to embryonic lethality and impaired T cell proliferation (Park et al., 2005). Indeed, deficiency of FADD also leads to a dysregulation of the cell cycle progression (Werner et al., 2006). Previous report highlighted that phosphorylation of FADD is associated with cell cycle regulation and cell proliferation (Alappat et al., 2005; Bhojani et al., 2005). FADD also participates in anti-oxidant and redox signaling (Cheng et al., 2014). Notably, FADD also attributes in autophagy signaling by interacting with DD of Atg5 to promote type II programmed cell death (autophagy) (Pyo et al., 2005). Moreover, FADD deficiency in T cells provokes autophagic signaling and subjected to caspase-independent cell death (Bell et al., 2008). The versatility of FADD has also been reported in RIP1- and RIP3-dependent necroptosis signaling (Han et al., 2011; Welz et al., 2011). Canonically, the DR stimulation in the presence of FADD proteolytically activates caspase-8 to cleave RIP1 and RIP3 in preventing necroptosis (Lin et al., 1999; Feng et al., 2007; Holler et al., 2000). Interestingly, FADD is now speculated to have an important role in inflammatory signaling and related disorders. Some reports advocate that FADD has been linked in the modulation of NF-κB and TLR mediated signals during infectious diseases, through interferon production (Ma et al., 2004; Salaun et al., 2007). Moreover, the regulation of FADD by E3 ubiquitin ligaes MKRN1 has been reported as an important regulator of its proapoptotic activity (Lee et al., 2012). Altogether, FADD has multiple regulatory functions and may serve to modulate both apoptosis and non-apoptotic signaling (Figure 7). Thus, understanding the cellular dynamics of FADD in the processes of cell death and survival may unravel the potential of this protein as a drug target to act against cancer.

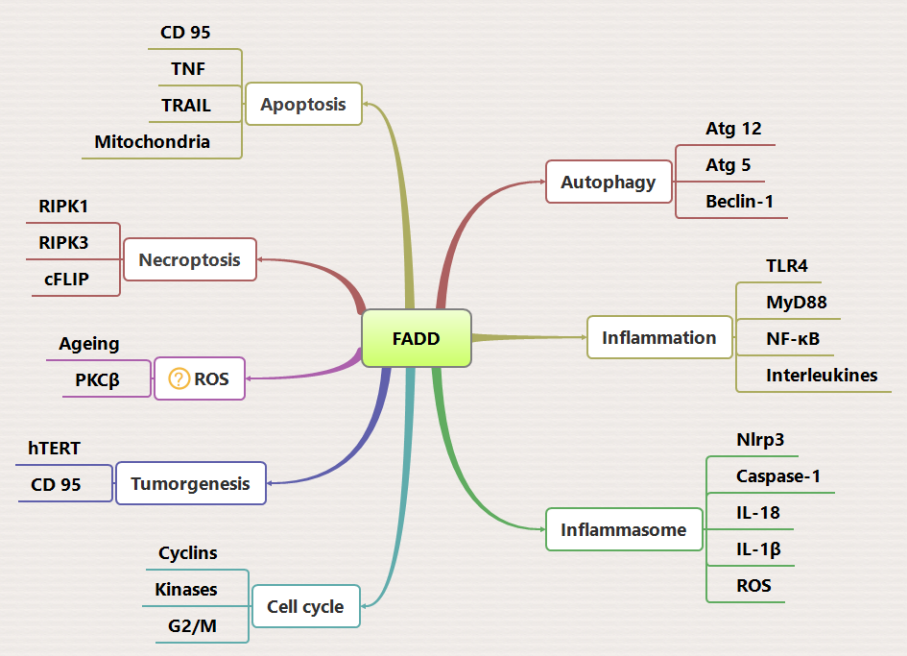

Figure 7. Multiple role of FADD in regulation of cellular signaling. The adaptor protein FADD has diverse role in regulation of various cellular processes including Apoptosis, Necroptosis, Autophagy, Inflammation, ROS signaling, Cell cycle and Tumorigenesis.

1.2.4 Structural organization of FADD

Human FADD is a 208 amino acid containing 22 kDa protein encoded by two exons located on chromosome 11q13.3 (Kim et al., 1996). It has two functional domains known as N-terminal Death Domain (DD) and the C-terminal death Effector Domain (DED) (Figure 8). The DD of FADD consists of 80 amino acids long six antiparallel amphipathic α-helices, which is a structural mimic of CD95 DD and responsible for homophilic interaction with death receptors (Chinnaiyan et al., 1995; Jeong et al., 1999; Scott et al., 2009). The DD of FADD also interacts with adaptor protein TRADD in TNFR-1 siganling, DR3, TRAIL receptors 1 (DR4) and 2 (DR5) to transduce extrinsic signaling of apoptosis (Hsu et al., 1996; Barnhart et al., 2003). The C-terminal DED of FADD interacts with DEDs of procaspase-8 or 10 to form DISC (Boldin et al., 1996; Muzio et al., 1996).

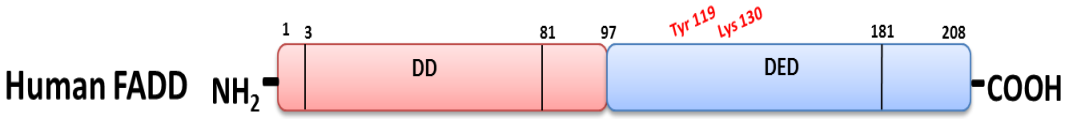

Figure 8. Structural organization of human FADD. The human FADD is consists of 208 amino acids and composed of N-terminal DD that interacts with activated death receptor and C-terminal DED allows to interact with apical caspases (procaspase-8 and -10).

The three-dimensional structure analysis of FADD reveals that DD and DED domains are oriented in an orthogonal tail to-tail fashion and each domain have a strong resemblance in the conserved backbone of six α-helices (Kim et al., 1996; Carrington et al., 2006). Earlier reports suggest that the DD of FADD carries a patch of positively charge residues (K110, R113, R114, R117, R127) in the vicinity of the α11/α12 interhelical loop to facilitate the interaction with DD of CD95/Fas receptor (Hill et al., 2004; Carrington et al., 2006) (Figure 9). In addition, the interfaces observation reveals that the first and sixth α-helix of FADD-DD participates in forming complex with DD containing proteins involved in cell death and inflammatory signaling (Scott et al., 2009; Yang, 2015). Furthermore, the *in vitro* study with the isolated DD of FADD show that Q169 and N171 residues facilitate the interaction with a DD of RIP1 protein and play an important role in regulating the necrotic activity of RIP1 (Park et al., 2013). In contrast, the NMR structural analysis revealed that each α-helix of the DED have some conserved hydrophobic and negatively charged residue such as Glu/ Asp or Asn at position 19 and RXDL motif at position 78-81 that is similar in nearly all DEDs (Figure 10). However, the distinguished heterogeneity in the second and third α-helix of FADD-DED highlight this molecule from remaining DEDs to serve as a ubiquitous platform in the formation of DISC (Eberstadt et al., 1998; Tibbetts et al., 2003) (Figure 9).

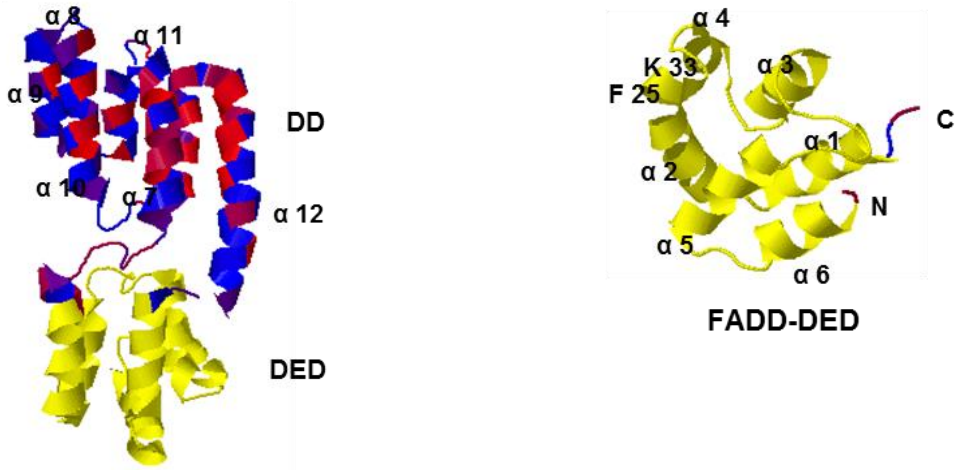

Figure 9. The three-Dimensional structure of human FADD. The DD of FADD carries a patch of positively charge residues (K110, R113, R114, R117, R127) in the vicinity of the α11/α12 interhelical loop to facilitate the interaction with DD of death receptor, FADD (PDB id- 2GF5 (intact FADD) & 1A1W (FADD-DED)).

Earlier mutagenesis and biochemical investigations have established the fact that individual domains of FADD cannot support the downstream signaling of apoptosis irrespective of DR activation. Indeed, the presence of DED is obligatory to the DD for a productive interaction with DRs (Carrington et al., 2006). In contrast, over expression of DED induces cell death independent of DR activation (Vanden Berghe et al., 2004).

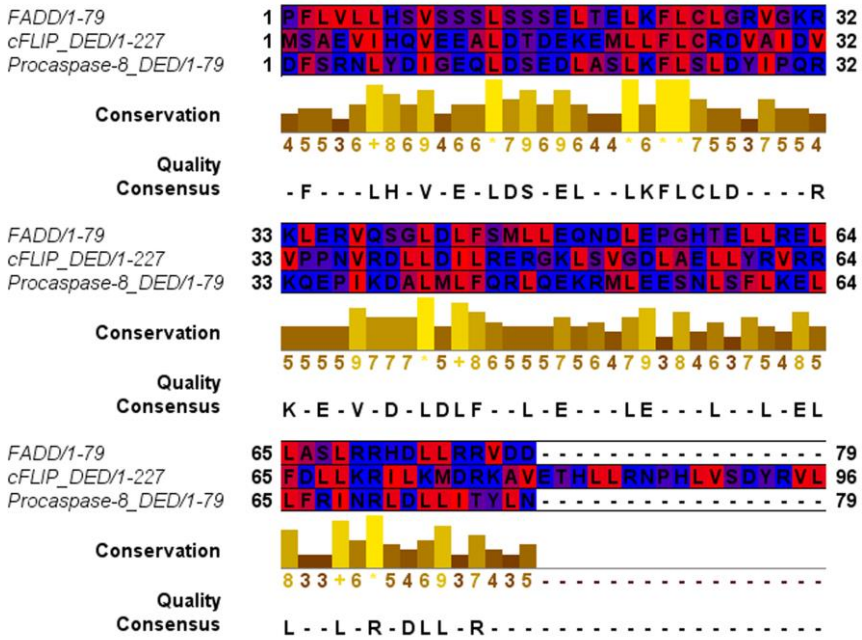

Figure 10. The conserved sequence of DED in FADD, cFLIP and pro-capsase-8. The sequence analysis show that each α-helix of the DED have some conserved hydrophobic and negatively charged residues, Blue clour shows hydrophic residues and red colour shows conserved residues. FADD (PDB id- 1A1W); $cFLIP_L$ (PDB id- 2BBR (MC 159)) and procaspase-8 (PDB id- 2K7Z).

However, the overexpression of FADD-DD has been reported to abrogate downstream signaling of activated Fas/CD95 and DR5 receptors in adherent cells (Zhang and Winoto, 1996; Sandu et al., 2006). The function of FADD was dependent upon its phosphorylation state. It has been demonstrated that FADD selectively phosphorylated at serine 194 located at C-terminus and regulates cell cycle progression (Matsuyoshi et al., 2006). Moreover, FADD is predominantly phosphorylated in G2/M stage and unphosphorylated in the G1 stage of the cell cycle (Scaffidi et al., 2000). However, the molecular mechanism of FADD phosphorylation is still unclear. The mutational analysis revealed that deletion or mutation in DED terminus of the FADD (FADD-DN) functions as a dominant negative in death receptor- signaling and found its inability to recruit caspase-8 (Chinnaiyan et al., 1996b). In contrast, overexpression of full-length FADD was found proapoptotic, due to the inherent ability of the FADD-DED to oligomerize and form a functional DISC upon activation of death receptor (Chinnaiyan et al., 1995; Siegel et al., 1998). Another study with the DED mutant of the FADD (deletion of 80-208) shows impaired activity of FADD in response to canonical death inducer and defect in T-cell proliferation (Zornig et al., 1998). Notably, the orthologs of human FADD have been noticed and characterized in the mouse and xenopus as well. The mouse FADD (mFADD) and xenopus FADD (xFADD) proteins shows 80% (205 amino acids) and 62% (188 amino acids) structural similarity with

human FADD (hFADD) respectively (Zhang and Winoto, 1996; Sakamaki et al., 2004). (Figure 11).

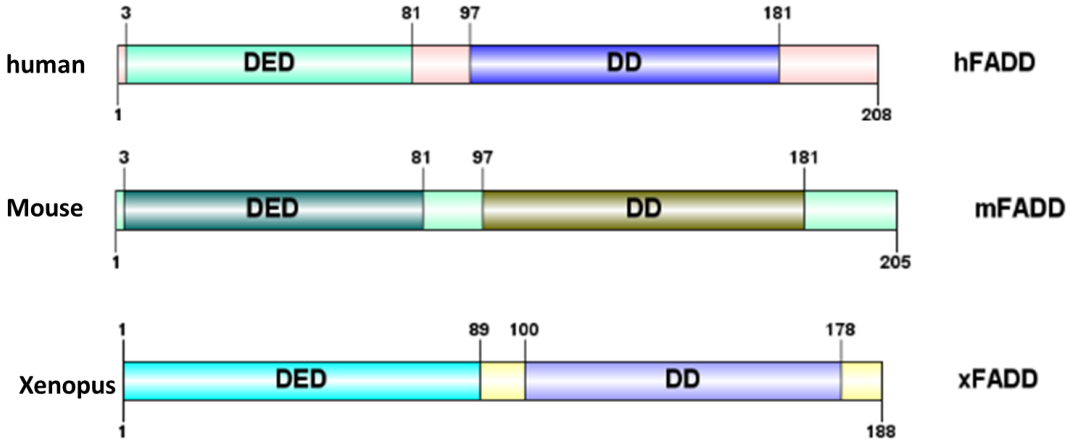

Figure 11. The orthologs of FADD in human, mouse and Xenopus. The mouse FADD (mFADD; 205 amino acids) and xenopus FADD (xFADD; 188 amino acids) proteins have 80% and 62% structural similarity with human FADD (hFADD; 208 amino acids) respectively.

Although, the ectopic expression of xFADD in mammalian cells induces apoptosis, however truncated (dominant negative) DED of FADD abrogates apoptosis in response to Fas ligand stimulation (Sakamaki et al., 2004). Thus structural analysis of FADD revealed that individual domains of this protein are crucial for normal cell proliferation and regulation of the cell cycle to proper development apart from its involvement in apoptotic signaling.

1.2.5 Sub-cellular localization of FADD

The cytosolic localization of FADD is obligatory for the DR signaling and DISC formation to induce apoptsis (Tourneur and Chiocchia, 2010). However, the post translational modifications of FADD identified its non-apoptotic function such as cell cycle progression, cell proliferation or genome surveillance (Screaton et al., 2003; Alappat et al., 2005). An ealier report suggests that FADD possess nuclear localization and nuclear export signals (NLS and NES) on the surface of DED to transduce nucleo-cytoplasmic shuttling (Gomez-Angelats and Cidlowski, 2003). In addition, mutation in Phenylalanine residue located at the 25th position of DED region or deleting the DED abrogates the nuclear translocation of FADD (Screaton et al., 2003). Some reports suggest that CK1α (casein kinase) and CK2β are the key candidates responsible for FADD phosphorylation, that directes them to translocate across the nucleus. Moreover, phosphorylation followed by nuclear translocation of FADD by CK1 is a primary event and later on CK2β retains the pFADD inside the nucleus to abrogate the death receptors signaling (Screaton et al., 2003; Vilmont

et al., 2015). Moreover, it was noticed that exportin-5, the nucleo-cytoplasmic shuttling protein, interacts with FADD to facilitate the import of phosphorylated FADD (pFADD) within nucleus, whereas Ser 194 mutation hinders FADD-exportin 5 interactions (Screaton et al., 2003; Zhang et al., 2004b). In addition, the nuclear localization of pFADD strengthens the anti-apoptotic activity of NF-κB to promote cell proliferation (Bhojani et al., 2005)Interestingly, some reports highlight that FADD mostly translocates in the high non-condensed transcriptionally active region of chromatin, but in the absence of DNA binding motifs FADD may not have direct role on transcriptional machinery (Cimino et al., 2012). Nevertheless, FADD may form a complex with the nuclear binding proteins to regulate genomic machinery. Earlier studies have shown that DED of FADD interacts with methyl-CpG binding domain protein 4 (MBD4) in the nucleus, however the downstream signaling of FADD-MBD4 is not well defined (Screaton et al., 2003). Notably, resolving the unidentified function of nuclear FADD will certainly help to utilize this protein as a tool to explore its nuclear significance apart from its distinguished role in transduction of apoptosis (Figure 12).

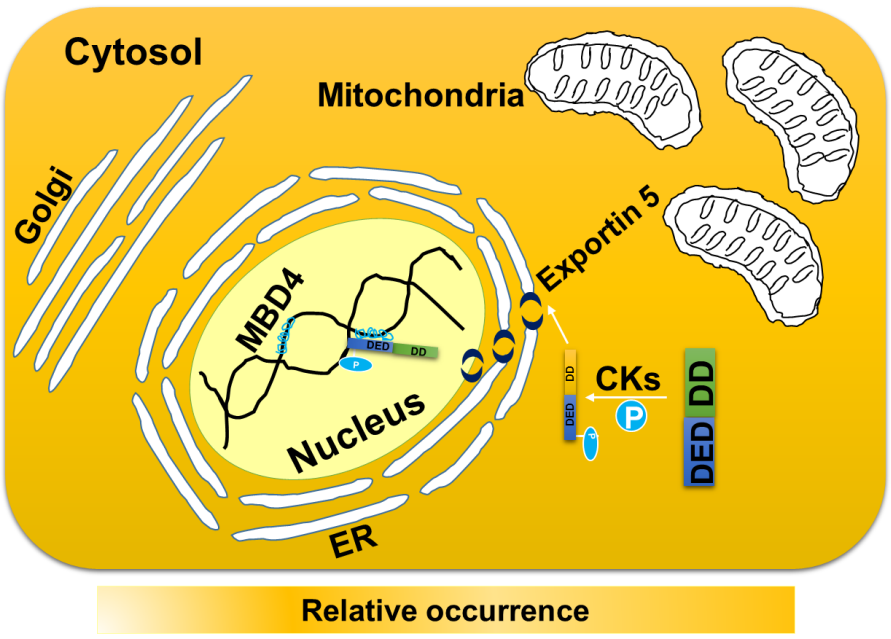

Figure 12 Cytosolic and nuclear sub cellular localization of human FADD. The sequence analysis of FADD shows two small stretches of nuclear localization and nuclear export signals (NLS and NES, respectively) on the surface of FADD-DED to transduce nucleo-cytoplasmic shuttling. The nucleo-cytoplasmic shuttling protein exportin-5, interacts with FADD to facilitate the import of phosphorylated FADD (pFADD) within nucleus. In contrast, the DED interacts with methyl-CpG binding domain protein 4 (MBD4) in the nucleus. ER- Endoplasmic Reticulum

1.2.6 Cellular Signaling of FADD in Apoptosis

Apoptosis is mainly triggered by activation of caspases through complex signaling, which includes extrinsic (death receptor) and intrinsic (mitochondrial dependent) signaling (Elmore, 2007; Taylor et al., 2008). The death receptor (DR) mediated apoptosis is initiated by binding of death ligands to their cognate receptors at the cell surface which triggers the signals for activation of initiator and effector caspases to execute cell death (Holler et al., 2003). The DR mediated apoptosis is mainly contributed by group of receptors like CD95, TRAIL-R1 and TRAIL-R2 which belong to the tumor necrosis factor-1 (TNF-1) receptor superfamily-1. These receptors have a characteristic death domain (DD) at its cytoplasmic tail, which homophilically interacts with cytosolic DD containing adaptor proteins FADD and TRADD for transducing the apoptotic signals. In contrast, the DED of FADD interacts with DED of executioner caspases-8 or 10 for the processing and activation of caspases-8. (Barnhart et al., 2003; Dempsey et al., 2003; Muppidi et al., 2004). The activated caspases-8 or 10 subsequently triggers cleavage of cytosolic protein Bid to the truncated Bid (tBid) that translocates the mitochondria for the initiation of intrinsic apoptotic cell death (Li et al., 1998; Kim et al., 2009). It is noteworthy that extrinsic and intrinsic signaling of apoptosis are interlinked by the activation of Bid (Wang, 2001). However, the in-depth molecular mechanism of cell death signaing associated with FADD remains elusive.

1.2.6.1 Fas (CD95/ Apo1/ TNFRSF6) in extrinsic cell death signaling

Fas receptor (FasR) belongs to the tumor necrosis factor receptor (TNFR) superfamily, that induces apoptosis signaling upon binding of its cognate ligand, CD95L (CD178/TNFSF6) (Guicciardi and Gores, 2009; Holler et al., 2003). The activated FasR interacts with FADD *via* death domain (DD) and exposes the DED for the oligomerization of procaspase-8 followed by formation of death-inducing signal complex (DISC) and subsequent processing and activation of downstream initiator and effectors caspases (Choi and Benveniste, 2004; Carrington et al., 2006). The stoichiometric analysis of DISC revealed that Fas, FADD and caspase-8/- or caspase-10 are probably organized in 3:3:3 ratio to form a trimeric structure (Siegel et al., 2000). The time-course analysis suggests that, within seconds of Fas stimulation FADD recruits procaspase-8 at the DISC, however the processing of procaspase-8 in p26/p28 and p18/p10 subunits requires between 5 to 10 minutes post stimulation, (Kischkel et al., 1995; Algeciras-Schimnich et al., 2002; Peter et al., 2007). Furthermore, the active caspase-8 differentially regulates the downstream signaling, either by rapid activation of executioner caspase-3 or by subsequent cleavage of

Bid protein into truncated Bid (tBid) to trigger the activation of the mitochondrion siganling (Arnoult et al., 2003; Lu et al., 2003). The tBid induces the Bax/Bak oligomerization and pro-apoptotic activity with opening of mitochondrion permeability transition pores (MPTPs) to release cytochrome c form mitochondria that subsequently activates the formation of apoptosome complex with the aid of cytosolic dATP (deoxyadenosine triphosphate) or ATP, apoptotic protease activation factor-1 (Apaf-1) and procaspase-9 (Susin et al., 1999; Chipuk and Green, 2005; Kroemer and Martin, 2005).

1.2.6.2 TNF-α signaling in cell death and survival

The TNF-α is an endogenous pleotropic cytokine that belongs to tumor necrosis factor super family with important functions in cellular immunity, cell differentiation, proliferation, inflammation and cell death (Wajant et al., 2003; Waters et al., 2013). The TNF-α exerts their biological effects through the cell surface TNF receptors (TNFR) that consists of ~ 80 amino acids long cytoplasmic death domain (DD) that is responsible for recruiting the downstream components of the death machinery (Waters et al., 2013). TNF-α is also known as type II trans-membrane protein with an intracellular 26 kDa amino terminus domain that can be cleaved by TNF-α-converting enzyme (TACE) into a 17 kDa soluble cytokine that is released into the extracellular space in cells (McGowan et al., 2008; Kornfeld et al., 2011). TNF-α can triggers various cellular responses indculsing cell survival, cell proliferation with transcription of pro-inflammatory genes and cell death (Waters et al., 2013b). TNF-α transduces its cellular response through interaction with two receptors, including TNF receptor type 1 (TNFR-1), which is expressed in most cells of the body, and TNF receptor type 2 (TNFR-2), which is mainly expressed in hematopoietic cells (Sakurai et al., 2003). The pleiotropic effect of TNF-α signaling is translated by binding with their receptors and interaction with cellular adaptor proteins. The activation of TNFR-1 allows a conformational change in its cytoplasmic DD tail to interact with DD containing adaptor protein TRADD (TNFR-associated death domain), rceptor interacting protein (RIP)-1, TNFR-associated factor (TRAF)-2 and -5 accompanied with cellular inhibitor of apoptosis (cIAP 1/2) to form the complex I (Wang et al., 1998); Sakurai et al., 2003). The formation of complex I directs the activation of NF-κB to transcribe upregulation of several anti-apoptotic genes such as Bcl-xL, A1/Bfl-1, (c-IAP) 1/2, X-chromosome-linked IAP (XIAP; also known as hILP) and cFLIP (Irmler et al., 1997, Brenner et al., 2015). Moreover, the formation of complex I also depends upon the cIAPs mediated ubiquitination of RIP1 protein (Bertrand and Vandenabeele, 2010; Kim et al., 2010). In addition, the transforming growth factor-β-activated kinase (TAK)-1/TAK-1

binding protein (TAB)-2/TAB-3 forms a complex that binds to ubiquitin residues on RIP1 and stabilizes complex I to activate NF-κB (Blonska et al., 2005; Broglie et al., 2010). In contrast, the deubiquitinylation of RIP1 by the enzyme cylindromatosis (CYLD) or cIAPs inhibitors subsequently disassociate RIP1 from complex I to form complex II in association with FADD and procaspase-8 to trigger cell death (Micheau and Tschopp, 2003; Tenev et al., 2011). The formation of RIP1-FADD-procaspae-8 complex II is constatntly under the survelience of anti-apoptotic protein cFLIP (Tenve et al., 2011). Recent report highlight that transcriptional regulation of cFLIP is linked to a number of growth and survival promoting signaling pathways, including NF-κB, MAPK/ERK and Akt (Marques-Fernandez et al., 2013). The evasion of apoptsis and activation NF-kB associated cellular signaling is an important component for cell proliferation in cancer (Karin and Lin Nature Immu 2002). Several reports highlight that NF-κB activity aids long term cell survival and susceptible to induction of apoptosis by chemotherapeutic drugs and radiation (Baldwin, 2001). Thus, activation of NF-κB and its anti-apoptotic function represents a major obstacle in successful cancer therapy.

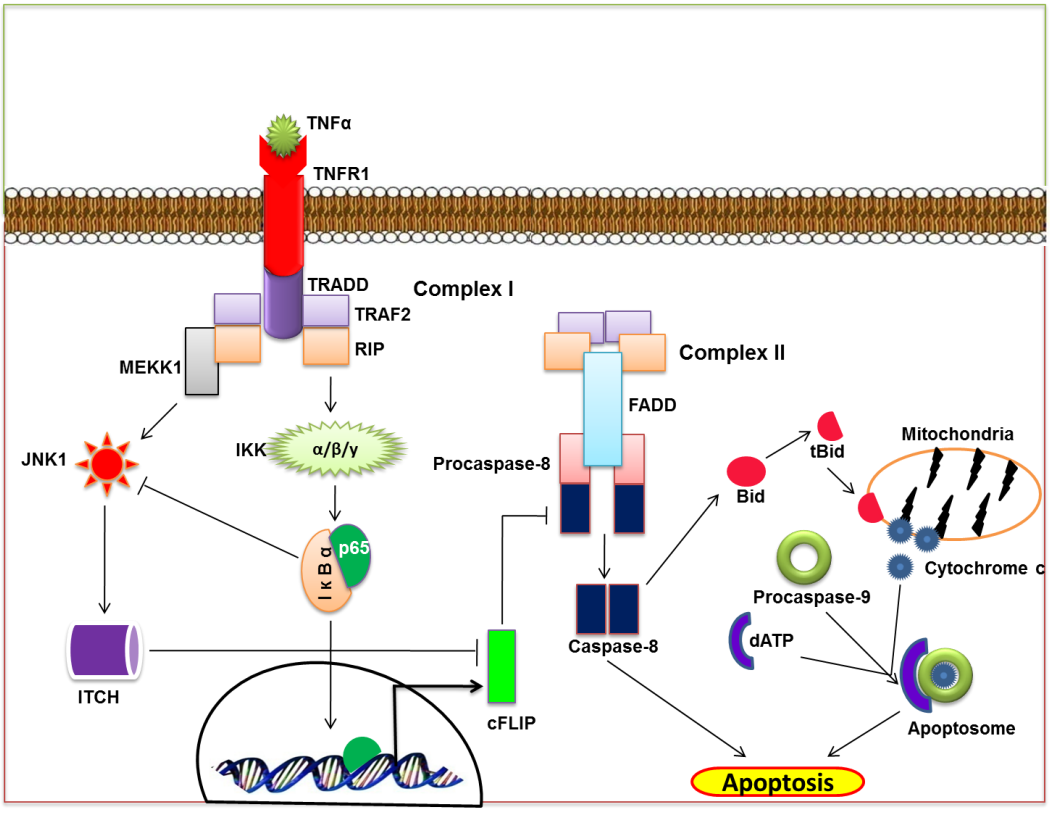

Figure 13. TNFR1 signaling. Ligation of TNF-α to TNFR1 induces its oligomerization and recruitment of adaptor protein TRADD, TRAF2, cIAPs and RIP1 to form complex I. The ubiquitin ligases TRAF2 and cIAPs allow ubiquitination of RIP1 to facilitate NF-κB activation and downstream transcriptional activation of $cFLIP_L$ to cell survival. The dissociation of RIP1 from complex I interact with FADD and caspase-8 to form complex II and execute cell deth.

1.2.6.3 TRAIL signaling

TRAIL (TNF-related apoptosis-inducing ligand) is the member of TNF-α super family that induces several signaling pathways. TRAIL induces signaling through TRAIL-R1 and TRAIL-R2 (TRAIL receptor) with the association of the FADD *via* the DD interation (Kuang et al., 2000; Peter, 2000; Werner et al., 2002). TRAIL-R1 (death receptor 4-DR4) and -R2 (death receptor 5- DR5) receptors contain a death domain (DD) in their intracellular portion that allow interaction of DD carrying protein to transduce death signals (Ehrhardt et al., 2003; Lahm et al., 2003). In contrast, the TRAIL-R3 and -R4 receptors are associated with cell survival and NF-κB activation due to absence (truncated) of DD (Degli-Esposti et al., 1997). Upon ligation of TRAIL ligand the TRAIL-R1 or -R2 receptors induces oligomerization of FADD by DD-DD interaction (Mongkolsapaya et al., 1999). Earlier reports have shown the role of FADD in Fas and TNF-R1 signaling for apoptosis (Chinnaiyan et al., 1996a; Varfolomeev et al., 1998). However the involvement of FADD in TRAIL signaling remains controversial. Some earlier reports suggest that in FADD deficient mouse embryonic fibroblasts (MEF) overexpression of DR4 fails to induce apoptosis in the presence of TRAIL; whereas, FADD expressing MEF efficiently induces apoptosis in response to TRAIL (Kuang et al., 2000; Yeh et al., 1998). It has also been shown that DR5 dependent formation of DISC oligomerises FADD and caspase 8 and sensitize TRAIL response to cell death in Jurkat T cells (Bodmer et al., 2000). However, the in-depth molecular mechanism of FADD and TRAIL associated cellular signaling is not well understood (Figure 14).

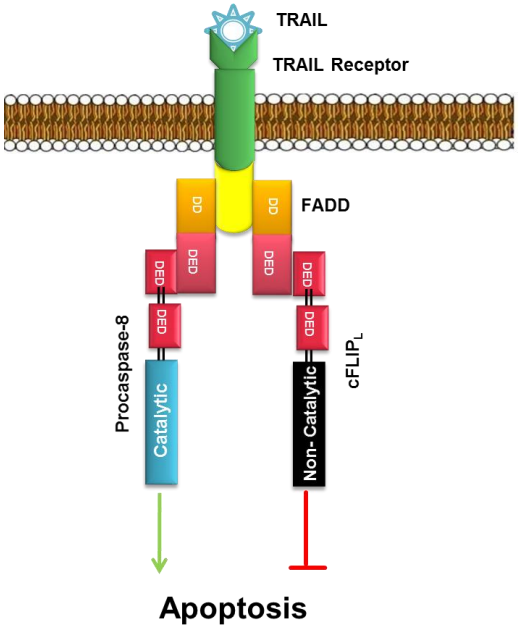

Figure 14 The TRAIL signaling of apoptosis. Binding of TRAIL ligand the TRAIL-receptor 1 (R1) or -R2 receptors induces oligomerization of FADD by DD-DD interaction to formation of DISC for induction of apoptosis. FADD provides binding site for both signaling molecules procaspase-8 and cFLIP to determine the cell death or survival.

1.2.7 RIP1 and FADD in cell death and survival

Receptor interacting protein1 (RIP1) is also an adaptor protein that allows the binding of death receptors such as TNF-R1 and TRAIL-R1/R2 (Dempsey et al., 2003; Sakurai et al., 2003). The importance of RIP1 in cellular homeostasis was unravelled when a deficiency of RIP1 in mice was found to be lethal after birth due to extensive apoptosis in both lymphoid and adipose tissue (Kelliher et al., 1998). Thus, it was clear that RIP1 helps in maintaining cellular homeostasis by regulating apoptosis machinery. However, it has been also shown that RIP1 may activate cellular survival signaling by the NF-κB pathways (Bertrand and Vandenabeele, 2010; Festjens et al., 2007). Previous report demonstrates that RIP1 deficient cells were unable to induce NF-κB activation stimulated by TNF-α (Kelliher et al., 1998). RIP1 also links TNFR1 to the caspase cascade, forming a complex II that is involved in pro-apoptotic signaling with the aid of FADD and procaspase-8 (Tenev et al., 2011; Festjens et al., 2007). Indeed, NF-κB activation is blocked by TNF-α mediated signaling through activation of capsase 8 and cleavage of RIP1 (Lin et al., 1999; Wang et al., 2008). Therefore, the stability of FADD-caspase-8 containing complex II strengthens apoptosis when complex I-mediated activation of NF-κB and expression of cFLIP remains poor (Figure 15). Thus, the cleavage of RIP1 and activation of caspases 8 may define a major regulatory role in determining the fate of cell death or survival in presence of TNFα. Upon TNFα stimulation, RIP1 gets autophosphorylated and polyubiquitinated on both Lys48 and Lys63. In addition, Lys48 polyubiquitination leads to degradation of the protein by proteasomes, while Lys63 linkages result in the activation of the IκB kinase (Kim et al., 2010). In fact, Lys 63 polyubiquitination of RIP1 is required for the TNFα-induced activation of NF-κB for cell survival signaling (Ea et al., 2006). Moreover, a point mutation of RIP1 on Lys377 (K377R) blocks polyubiquitination by K63-linked polyubiquitin chains and prevents the recruitment of IκB kinase, IKKβ and TAK1 complex to the TNF receptor and thereby inhibiting NF-κB activation (Sakurai et al., 2003; Ea et al., 2006). Therefore, polyubiquitination of RIP1 is essential for the activation of IKKβ that phosphorylates IκB, an inhibitor of NF-κB, and targets it for degradation by the proteasomal pathway (Wertz and Dixit, 2010). Furthermore, NF-κB is then liberated from the inhibitory complex and is translocated to the nucleus where it initiates target gene transcription involved in immunity, inflammation and survival (Chen, 2005). The mechanism by which RIP1 induces NF-κB activation is dependent on RIP1 ubiquitination and activation of an intermediate IκB signaling complex is needed to explore.

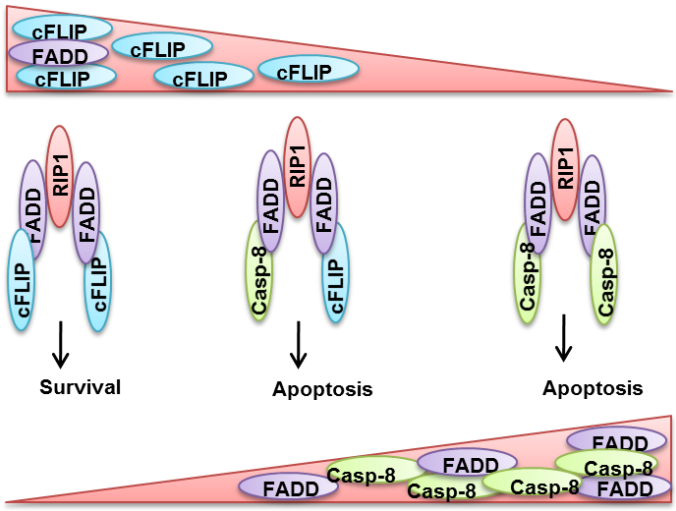

Figure 15. Interaction of FADD and RIP determines fate of cell death and survival signaling. TNF-α triggers signaling of apoptotic cell death via formation of complex II. FADD stabilizes RIP1 and procaspase-8 associated pro-apoptotic complex II. The strong interaction between DDs of FADD and RIP1 favours apoptotic instigation. Indeed, the formation of a DD complex between FADD, RIP1 and procaspase-8 remains under the surveillance of anti-apoptotic protein cFLIP.

1.2.8 Role of FADD in Autophagy

The process of autophagy generally referred to as "self-eating" process, tends to degrade the unwanted cytosolic constituents by transporting them to lysosome in order to protect the stress induced cell death (Levine and Klionsky, 2004; Levine and Yuan, 2005, 2004). Basal level of autophagy helps to maintain homeostasis in normal conditions, while during stress conditions autophagy also supports cell survival (Bergamini et al., 2004; Levine and Klionsky, 2004). In contrast, apoptosis and autophagy are interlinked by key regulatory molecules responsible for cell death and survival (Mukhopadhyay et al., 2014). The autophagic protein Atg5 has been reported in the dual regulation of autophagy and cell death (Pua et al., 2007). Mechanistically, the Atg5 conjugates with Atg12 and form an autophagosome as a punctuate structure that is known as a hallmark of autophagy induction (Tanida et al., 2004). In the setting of autophagy the DD of FADD interacts with Atg5 and commences autophagic cell death (Pyo et al., 2005; Thorburn et al., 2005; Bell et al., 2008). The in-depth analysis revealed the presence of a Lys residue in the C-terminal region of Atg5 that is common for conjugation with either Atg12 or FADD for respective downstream cell signaling (Pyo et al., 2005). Previous reports advocate that FADD and caspase-8 together regulate the autophagic signaling for proper T cell proliferation (Li et al., 2006; Pua et al., 2007). Mechanistically, the mitogenically activated T cells induced the interaction of FADD to Atg5:Atg12 complex resulting in caspase-8 activation accompanied

with autophagic cell death (Pua et al., 2007). The depletion of FADD enforced T cells to undergo hyperautophagy and activate RIP1-dependent necroptotic cell death, independent of caspase-8 activation (Walsh and Edinger, 2011). However, re-expression of full-length FADD in FADD-/- MEFs restores the basal level autophagy induced by serum deprivation (Bell et al., 2008). The above findings suggest that the presence of FADD and concurrent activation of caspase-8 may restrict hyperautophagy and necroptotic death to channelize the commencement of apoptotic cell death (Tait et al., 2014). Although, the cross-talk between apoptosis and autophagy may vary with cell type and the nature of stimulus, a better understanding of regulators involved in both the pathways may be useful to design a common strategy to regulate both the processes (Figure 16).

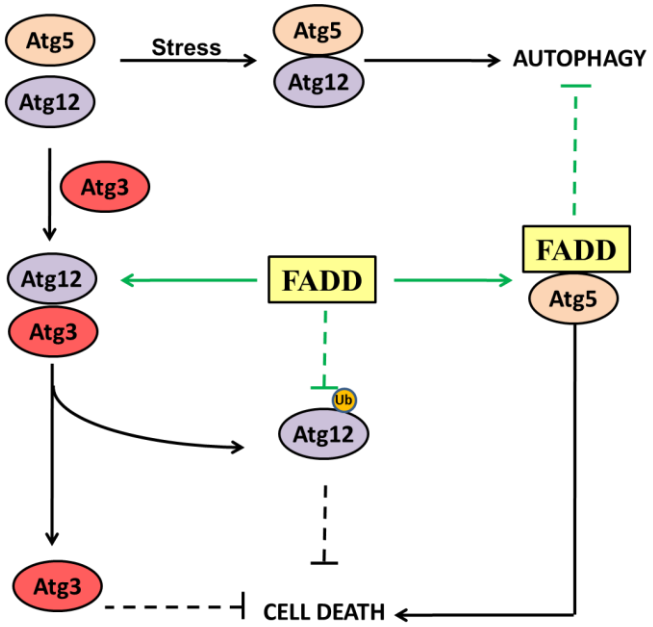

Figure 16 Role of FADD in autophagy. The Lys residue at the C-terminal region of Atg5 provides common binding site for Atg12 or FADD for interaction and respective downstream cell signaling. The interaction of Atg5 with Atg12 initiates formation of an autophagosome for autophagy induction. In contrast, the DD of FADD interacts with DD of Atg5 and commences autophagic cell death.

1.2.9 Role of FADD in Necroptosis

Necroptosis is a form of caspases-independent cell death by the involvement of necrotic proteins RIPK1/3 and under apoptotic deficient conditions (Vanden Berghe et al., 2014). Importantly, some of the apoptotic components are deeply involved in the formation of the necroptotic complex in cell death (Degterev et al., 2005; Christofferson and Yuan, 2010; Vandenabeele et al., 2010b). Necroptosis signaling regulates a variety of cellular and pathological processes ranging from development to the pathogenesis of the immune regulatory cells (Han et al., 2011). The signaling of necroptosis may be induced by the

plethora of stimuli, including, TNF-α, Fas, Toll-like receptors (TLRs) and sensors of viral DNA and RNA (Kalai et al., 2002; Upton et al., 2010). In addition, genotoxic agents and related cellular stress can also induce necroptosis (Davis et al., 2010; Tenev et al., 2011). In contrast, the dissociation of RIP1 form membrane bound TNFR1 complex forms proapoptotic 'ripoptosome' complex with clustering of RIP3 I in association with FADD and caspase-8 to divert apoptotic to necroptotic signals (Degterev et al., 2005; Vandenabeele et al., 2010a; Han et al., 2011). Although, FADD and caspase-8 are common components in ripoptosome to direct necroptosis, they reportedly block necrotic cell death signals (Cho et al., 2009; He et al., 2009; Christofferson and Yuan, 2010; Vandenabeele et al., 2010a). Some recent reports suggest that deletion of RIP3 completely restores the proliferation of T cells bearing mutation in FADD and rescues caspase-8-/- T cells from induced necroptosis (Ch'en et al., 2011; Lu et al., 2011). Furthermore, the stability of RIP1-RIP3 complex and associated necroptosis signaling is constantly challenged by FADD and caspase-8, as inhibiting the expression of caspase-8 or FADD leads to cardiovascular defect and impaired blood cell formation in mice resulting in embryonic lethality (Vandenabeele et al., 2010b; Kaiser et al., 2011; Lee et al., 2012; Vanlangenakker et al., 2012; Welz et al., 2011; Wu et al., 2012). Furthermore, FADD has also been reported in suppressing the RIP3-mediated chronic intestinal inflammation and necrosis in the epithelial cell (Welz et al., 2011). In contrast, Fas signaling mediated suppression of RIP3 by caspase-8 or FADD facilitate T cell clonal expansion (Bell et al., 2008; Lu et al., 2011). Moreover, FADD has been reported to enhance the ubiquitin activity of E3 ligase TRIM21 to protect the cells against viral induced cytokine production (Young et al., 2011). Importantly, the E3 ubiquitin ligase MKRN1 regulates the ubiquitination of FADD and RIP1–RIP3 complex formation (Lee et al., 2012). In addition to FADD and caspase-8, the level of cFLIP defines a major role in necroptosis by participating in formation of ripoptosome assembly (Tsuchiya et al., 2015). However, in the absence of active caspase-8 the process of apoptosis or necroptosis are completely blocked by cFLIP (Feoktistova et al., 2011; Tenev et al., 2011). Thus, FADD mediated regulation of necroptosis signaling could provide an opportunity to define the fate of cell in pathological consequences (Figure 17).

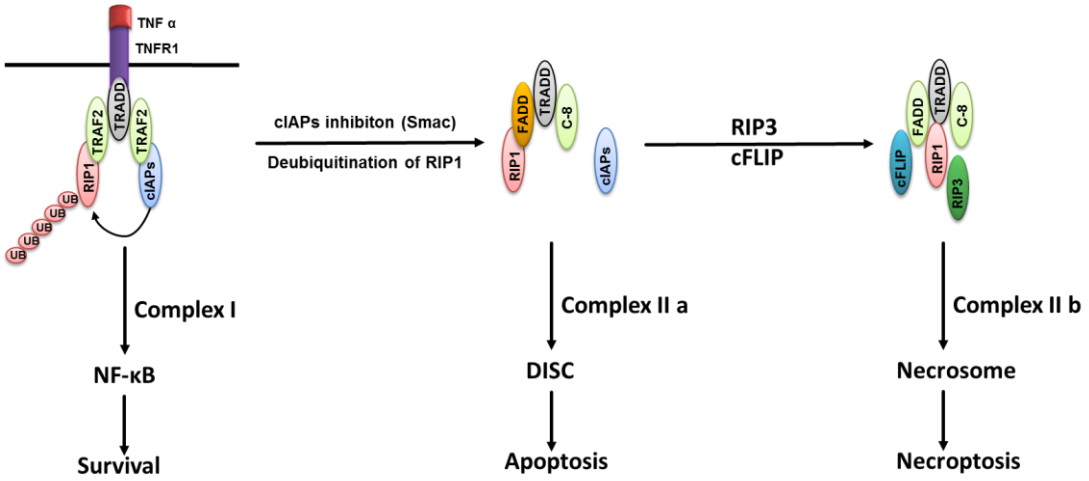

Figure 17. Role of FADD in Necroptosis. The expression of FADD, procaspase-8 and cFLIP are important to balance the shift from apoptosis or necroptosis signaling. The inhibition of cIAPs stabilizes RIP1 and dissociates it from TNFR1 complex 1 to form proapoptotic 'ripoptosome' complex IIa with the association FADD and pro-caspase-8. In addition, the expression of RIP3 and cFLIP are critical to regulate necroptosis. In the absence of active caspase-8, the RIP3 interacts with RIP1 and forms complex IIb to commence necroptosis signaling. However, cFLIP completely blocked the apoptosis and necroptosis signaling.

1.2.10 Role of FADD in Inflammation

Inflammation is a protective and essential immune response against any external or internal stimuli that disturb the cellular homeostasis (Coussens and Werb, 2002). The inflammatory response in the vicinity of tumor is likely caused by necrotic cell death in the core of tumor mass due to lack of oxygen and nutrients (Cho et al., 2009). These inflammatory events are accompanied by the production of cytokines, chemokines and growth factors, favoring increased cellular proliferation (Lin and Karin, 2007) The hyperactivation of inflammatory cytokines establish a tumor microenvironment and evade the mechanism of cell death to promote cell survival (Coussens and Werb, 2002; de Visser et al., 2006). The cells of tumor microenvironment interact with each other by secreting and sensing inflammatory cytokines and chemokines (Gao et al., 2007; Lin and Karin, 2007). Several cytokines such as IL-1α, IL-1β, IL-6, IL-23, IL-18, IL-33 and TNF-α has been reported as critical for both inflammation and cancer progression (Becker et al., 2004; Lin and Karin, 2007; Grivennikov et al., 2010). However, the unwanted inflammatory stress is regulated by various sets of genes responsible for cell death and survival (Lin and Karin, 2007; Zhou et al., 2010). Consistent with this, FADD has also been linked in the regulation of inflammatory activation apart from its role in regulating apoptosis, necroptosis and autophagic signaling (Rensing-Ehl et al., 1995; Desbarats et al., 2003; Schock et al., 2015). A recent report suggests that the FADD depleted myeloid cells (mFADD-/-) induce the production of inflammatory cytokines and with elevated B cell populations (Schock et al., 2015). Several reports highlight TLR4 signaling as a major

transducer of inflammatory response in various tumor models (Lien et al., 1999; Aliprantis et al., 1999; Kawai and Akira, 2007, 2010). Mechanistically, upon sensing the pro-inflammatory cytokine LPS the TLR4 receptor recruits adaptor proteins MyD88 and IRAK (Kawai and Akira, 2007). In addition, the DD of MyD88 facilitates its interaction with DD of IRAK to further activate NF-κB and transcription of target genes (Daun and Fenton, 2000). In contrast, the DD of FADD interact with DD of MyD88 and hinders IRAK association to MyD88, hence FADD is able to regulate the TLR4 activation and associated signaling of NF-κB (Aliprantis et al., 2000). However, the loss of FADD enhances MyD88-IRAK1 interaction, suggesting that FADD balances the IRAK1 binding to MyD88 in response to TLR4 activation (Aliprantis et al., 2000; Zhande et al., 2007). In contrary, some reports suggest that FADD may activate NF-κB signaling during conjugation with MyD88 and IRAK (Chaudhary et al., 2000; Hu et al., 2000; Schaub et al., 2000). However, the magnitude of this activation and the nature of stimulus may also have a profound influence in the downstream signaling (Bannerman et al., 2002; Duckett, 2002). Furthermore, the DD interaction of FADD and MyD88 suppresses the activation of JNK and PI3K pathways, independent of LPS stimulation (Zhande et al., 2007). In contrast, an interesting report proposes the involvement of FADD in the non-apoptotic activation of inflammatory cytokines IL-1β in bone marrow derived dendritic cells (BMDCs) upon Fas stimulation (Bossaller et al., 2012). However, in the absence of FADD the stimulation of Fas failed to produce IL-1β, suggesting that FADD may differentially regulate Fas signaling of apoptosis and inflammation (Bossaller et al., 2012). Similarly, a recent report highlights the role of FADD in caspase-8 mediated activation of IL-1β in LPS stimulated BMD cells (Moriwaki et al., 2015). Recent reports highlight that the Inflammasome activation together with activated NF-κB, triggers canonical maturation of IL-1β to support tumor progression (Latz et al., 2013; Abderrazak et al., 2015). Although, NF-κB activation is known to provokes the expression of proinflammatory cytokines. Thus targeting NF-κB may be considered as a regulatory check point of inflammatory signaling mediated by TLRs and MyD88 protein. Collectively, from the above finding it could be considered that FADD can play an important role in regulation of NF-κB activation and inflammation associated cancer progression (Figure 18).

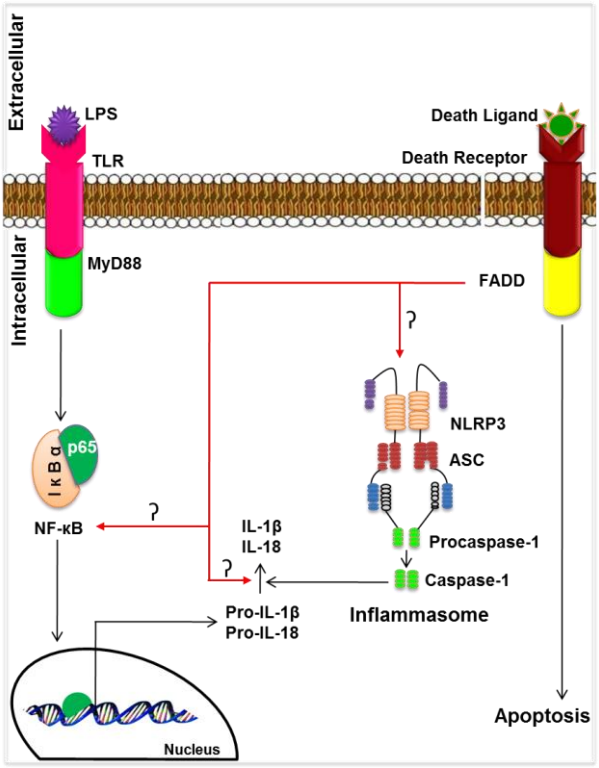

Figure 18 Role of FADD in inflammatory signaling. The pro-inflammatory cytokine LPS binds to the TLR4 receptor and recruits adaptor proteins MyD88 and IRAK to activate NF-κB signaling. The NF-κB induced activation of proinflammatory cytokines pro-IL-1β and pro-IL-18 needs a secondary trigger from Inflammasome complex. The inflammasome mediated activation of caspase-1 triggers processing of pro-IL-1β and pro-IL-18 into mature forms. The role of FADD in regulation of IL-1β and IL-18 remains elusive.

1.2.11 Role of FADD in cancer and therapeutic intervention

FADD is the central adaptor molecule and essential for the death receptor mediated pathways for the initiation of apoptosis. Previously, it was noticed that altering the expression of FADD in T cells imparts resistance against Fas ligand (FasL) induced apoptosis and promotes cell proliferation (Newton et al., 1998). In addition, a subsequent study on mice carrying dominant negative FADD shows the development of thymic lymphoma with the progression of age and established the role of FADD in tumor suppression (Newton et al., 2000). A large growing body of evidence highlighted that inadequate level of FADD may lead to greater resistance to death receptor-mediated apoptosis and may contribute to tumor progression in both mice and human (Tourneur et al., 2003; Tourneur and Chiocchia, 2010). However, some reports highlight that FADD deficient cells may resist to Fas signaling, but can induce apoptosis in response to cytotoxic agents (Newton and Strasser, 2000). The tumor suppressor function of FADD critically relies on the phosphorylation status and cellular localization (Alappat et al., 2005; Osborn et al., 2007). For instance, the phosphorylation of FADD in tumors triggers its nuclear localization and G2/M arrest (Shimada et al., 2005). Recently it was observed

that phosphorylated FADD serves as a prognostic biomarker in humans with early-stage glottic carcinomas (Schrijvers et al., 2012). An earlier study demonstrated that gene delivery of fused FADD with human telomerase reverse transcriptase (hTERT) promoter effectively induces apoptosis in malignant glioma cells while keeping normal cells unaffected (Komata et al., 2001). The recent advancement in cancer therapy opens the possibility of genetic manipulation to target specific genes that may control various pathological consequences such as tumorigenesis and inflammatory disorders (Mulligan, 1993). Earlier reports highlight that the adenovirus or retrovirus mediated transfer of FADD gene potently induces apoptosis in human malignant glioma cells (Kondo et al., 1998; Shinoura et al., 1998). In contrast, it has been well established that propagating inflammation is one of the major causes of evading apoptosis (Pope, 2002; Pommier et al., 2002). Some reports highlight the potential of FADD in regulation of inflammatory arthritides (Okamoto et al., 2000; Peng, 2006). Interestingly, the overexpression of FADD in synoviocytes of patients with rheumatoid arthritis (RA), induces apoptosis (Okamoto et al., 2000). In addition, the transfection of FADD into RA synoviocytes enhanced apoptosis by activating the caspase cascade of these cells both in vitro and in vivo, independent of Fas antigen (Kobayashi et al., 2000). Moreover, it can be hypothesized that the expression of FADD could be used as a prognostic factor for poor response to chemotherapy (Tourneur, L. et al., 2003; Tourneur, L. et al., 2004). Thus, implicating the above mentioned approaches in delivering the FADD in tumor cells could be useful to explore protein as an anti-cancer drugs. It is progressively clearer that gene or protein targeted therapy would be more effective than conventional chemo- or radiotherapy for cancer treatment. The aim of the present study is to provide a novel approach in terms of molecular mechanism and induction of apoptosis in cancer cells which in turn could offer enormous potential for cancer therapy.