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Cytokine-induced tumor suppressors: a GRIM story

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Abstract

Cytokines belonging to the IFN family are potent growth suppressors. In a number of clinical and preclinical studies, vitamin A and its derivatives like retinoic acid (RA) have been shown to exert synergistic growth-suppressive effects on several tumor cells. We have employed a genome-wide expression-knockout approach to identify the genes critical for IFN/RA-induced growth suppression. A number of novel Genes associated with Retinoid-Interferon-induced Mortality (GRIM) were isolated. In this review, we will describe the molecular mechanisms of actions of one GRIM-19 which participates in multiple pathways for exerting growth control and/or cell death. This protein is emerging as a new tumor suppressor. In addition, GRIM-19 appears to participate in innate immune responses as its activity is modulated by several viruses and bacteria. Thus, GRIMs seem to couple with multiple biological responses by acting at critical nodes.

Keywords

cytokines; tumor suppressors; apoptosis; transcriptional inhibition and immune response

Introduction

Apoptosis, a cell suicide mechanism, controls cell numbers in tissues; and protects the host by eliminating potentially-dangerous cells [1]. Two major pathways of apoptosis, the extrinsic and the intrinsic (Fig. 1), dismantle cellular architecture in response to various death stimuli by activating caspase-driven proteolytic cascades.[2]. Most caspases, a class of cysteine proteases, exist in a dormant state, owing to the presence of an N-terminal prodomain that prevents their spontaneous activity. Signal-induced association of caspases via homotypic domain interactions results in autocatalytic cleavage and release of prodomains, which unleashes their lethal effects on cells. In the extrinsic pathway, extra-cellular protein ligands such as TNF, FasL, TRAIL etc bind to specific cell surface receptors [3,4] and initiate the formation of the death-initiating signaling complex at the intra-cellular receptor surface, which involves either caspase-8 or caspase-10. A number of death receptors (DRs) have been characterized so far: DR1 (also known as TNFR1, CD120a, p55 and p60), DR2 (also known as FasR, APO1 and CD95), DR3 (also known as APO3, LARD, TRAMP and WSL1), DR4 (also known as APO2, TRAILR1), DR5 (also known as TRAILR2, KILLER and TRICK2, DR6, EDAR and NGFR [3,4]. These receptors possess a cytoplasmic death domain (DD) of approximately 80 amino acid residues. The death domain is a converging point for the assembly

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of scaffold proteins that will stage the first event in caspase activation (i.e. initiation). Autocatalytic activation of these either caspase-8 or caspase 10 (depending on the receptor/ligand) is followed by cleavage of the next layer of caspases, which then activate other caspases and/or cleave cellular proteins required for viability.

The intrinsic pathway is activated by various forms of stress inducers like γ -rays, *uv*-rays, cytotoxic drugs, removal of cytokines *etc*, and requires mitochondria. The latter harbor a number of cytotoxic protein factors required for initiating apoptosis in association with certain other cytoplasmic proteins including caspases. Thus, mitochondrial permeability transition [5] occurs invariably in the intrinsic death pathway. Following the opening of permeability transition pores (PTP), in response to death stimuli, molecules such as cytochrome-c escape from mitochondrion into the cytoplasm. Cytochrome-c associates with a scaffold protein, apoptosis-activating factor-1, which aids in the recruitment of caspase-9 into this complex in the cytoplasm. This complex, called the apoptosome, in presence of dATP, activates Caspase-9, which in turn activates Caspase-3 leading to the later events involved in cell death [6,7]. The mitochondrial membrane permeability is controlled by proteins belonging to the Bcl2 family that include both activators and inhibitors of PTP. All members of this family, although genetically distinct, possess characteristic Bcl2-homology (BH) domains [8,9]. Four types of BH domains viz., BH1, BH2, BH3 and BH4 have been identified in this protein family. Although not all domains are present in all members, all pro-apoptotic members invariably contain the BH3 domain. Some proteins which possess only BH3 domains are exclusively pro-apoptotic in nature [8,9]. The relative dynamics of pro- and anti- apoptotic members of the Bcl2 family control cell fate in response to activators of the intrinsic pathway e.g. Bcl2 favors cell survival while Bax promotes cell death [10–12]; Bcl-X_L is an inducer, whereas Bcl-X_S is an inhibitor of apoptosis [13]. Some pro-apoptotic members of Bcl2 family, such as BAD, are kept in check by growth factor-induced phosphorylation, which prevents their association with mitochondrion [14–18]. Others such as the Bid protein, is cleaved by caspase-8 to generate a truncated cell death-activating Bid (t-Bid) protein [19–21].

The intrinsic pathway is also activated in response to DNA damage, hypoxia etc. The p53 protein is a sensor of cellular stress and turns on the intrinsic pathway [22,23] by inducing the expression of pro-apoptotic members of the Bcl-2 family while repressing anti-apoptotic Bcl-2 members and cellular inhibitors of apoptosis proteins (CIAPs) [5]. Some p53-inducible pro-apoptotic Bcl-2 proteins include BAX, Noxa, PUMA, BID and genes which cause an increase in ROS and damage mitochondrion [24]. Some studies show a direct association of p53 with Bcl-2 proteins in mediating apoptosis, independently of its transcriptional activity [25]. In general, the intrinsic and extrinsic pathways culminate in the activation of Caspase-activated DNase (CAD) [26] and generation of pro-apoptotic peptides from Bcl-2 and Bcl-X_L proteins [27,28]. Although many death signals employ at least one of these pathways in later stages of cell death, their upstream factors are quite often different. Thus, the Bcl-2, Caspase and IAP proteins operating at the terminal stage of apoptosis form the “core death machinery”. This machinery is controlled by a number of exogenous factors. In contrast to chemotherapeutics, cytokine-induced cell death mechanisms are poorly defined. In this review we will describe the action of an IFN-regulated novel cell death mediator.

Interferon signaling in growth control

Cytokines and vitamins play a major role in suppressing neoplastic cell growth by participating in a tumor-surveillance system *in vivo*. The IFN family of cytokines protects hosts against infectious agents and neoplastic cell growth by two mechanisms: 1) by directly activating gene expression cascades in the target cell; and 2) by promoting specific immunity against pathogen or tumor-derived antigens [29–32]. They are the first-line effectors of innate defenses. The cancer cell- or pathogen- derived protein(s) in an infected cell, in turn, tries to ablate these

cytokine-induced regulatory effects [33–46]. Thus, a battle between cytokine-induced response and the pathogenic cell defines an effective outcome. One of the first effects of cytokines is to eliminate the infected or neoplastic cells. Two types of mechanisms viz., inhibition of cell cycle and induction of cell death limit the number of affected cells. Cell death occurs by three modes: 1) apoptosis; 2) autophagy; and 3) necrosis [47–52]. In this review, we will primarily discuss the molecular mechanisms of actions of novel cell death effectors discovered in our laboratory, the GRIMs.

As already said, IFNs are critical regulators of anti-viral, anti-microbial, anti-tumor, cellular differentiation and immune response activities [53–55]. IFNs signal through the JAK-STAT pathway [56,57] to upregulate the expression of genes involved in the above mentioned biological activities. Two classes of transcription factors viz., Interferon gene-Regulatory Factors (IRFs) and STATs [58,59] participate in canonical IFN response. The former regulate IFN genes and some ISGs, while the latter primarily regulate ISGs. However, some IRFs have been implicated in the induction of certain IFN-regulated genes [60–62]. These factors cross-regulate each other and form complexes with other factors to deliver distinct biological response(s) based on the elements present in the ISG promoter [62]. For example, the Interferon-Stimulated Response Element (ISRE) binds a heteromeric transcription factor, ISGF3, comprising of IRF9, STAT1 and STAT2, for inducing transcription of certain ISGs [56,57]. In a similar manner, binding of activated STAT1 dimer to Gamma-Activated Sites (GAS) of some ISGs stimulates gene expression in response to IFN- γ [63,64]. Cytokine-induced STAT-driven responses are controlled by feedback mechanisms, which regulate gene expression. The first mechanism involves dephosphorylation of activated STAT proteins by a nuclear tyrosyl phosphatase TcPTP [65,66]. The second mechanism involves proteins that inhibit the DNA binding activity of activated STAT proteins, *e.g.*, the binding of PIAS1 to STAT1 or PIAS3 to STAT3 [67,68]. The third mechanism involves STAT-induced proteins called Suppressor of Cytokine Signaling (SOCS) proteins that bind to and inhibit JAKs *e.g.*, SOCS1 against STAT1 [69–71].

Consistent with their growth-suppressive actions, IFN receptors and the transcription factors regulated by them play important roles in eliminating neoplastic growth [32,72–76]. For example, mutations in the human IRF1 gene has been linked to the development of myeloid leukemias [77]; deletion of the IRF8 gene causes a CML-like disease in mice [78], loss of IRF8/ICSBP expression occurs in CML patients [79], and re-expression of IRF8 suppressed BCR-ABL oncogene-induced CML-like disease in mouse models [80]. The *Ifngr*^{-/-} and *Stat1*^{-/-} mice are highly susceptible to chemical carcinogenesis [75,76]. Although a great deal is known about the importance of upstream signaling molecules in tumor development, the precise mechanisms of anti-tumor actions are not fully clear.

IFNs were the first cytokine therapeutics introduced into clinics for treating many viral diseases, malignancies or syndromes [81]. The anti-tumor activity of IFNs as a single agent is comparable to that of many currently used therapeutics. In some cases, insensitivity to IFN-induced antitumor effects could be overcome by combining with other agents such as cell-differentiating agents, the retinoids. This probably is due to the observation that most tumors stay in a dedifferentiated state.

Retinoid signaling in growth control

Retinoids are a group of vitamin A-related natural and synthetic compounds that regulate cell growth, differentiation and embryogenesis in vertebrates [82]. All *trans* retinoic acid (ATRA or RA), a natural metabolite, can drive all functions of vitamin A except vision [82]. Deprivation of dietary vitamin A results in an incidence of cancers in experimental animals, which was reversed by RA treatment [83,84]. Retinoids employ two structurally similar, but

genetically distinct, classes of nuclear transcription factors *viz.*, Retinoic Acid Receptors (RARs) and Retinoid X Receptors (RXRs) to drive the expression of genes that possess the Retinoic Acid Response Elements (RARE) [85–88]. The RAR:RXR heterodimers bind to RARE constitutively, whose transcriptional activity is held in check by several co-repressor molecules. Binding of RA to the RAR induces a conformational change that allows the recruitment of nuclear receptor co-activators, which promote transcription. A number of isotypes and subtypes of RARs and RXRs (α , β and γ) participate in retinoid-induced transcription in a gene- and cell- specific manner. Most RAR and RXR genes are constitutively expressed except for the RAR β gene whose expression is up-regulated by RA-treatment [85, 87]. Mutations in the RAR and RXR genes occur rarely. The expression of RAR β gene is inhibited in some cancers and its re-expression restores growth arrest in response to RA [89]. Transgenic mice expressing anti-sense RAR β gene develop lung cancers; although the exact mechanism(s) is/are unclear [90]. One of the well-defined mutation that involves RARs is a reciprocal translocation that occurs between the RAR α (human chromosome 17) and the promyelocytic leukemia (human chromosome 15) in some forms of acute promyelocytic leukemia (APL) [91,92]. Despite this translocation the resultant fusion protein, PML-RAR α , responds to RA treatment and leukemic cell growth is successfully controlled in APL patients [93,94].

Crosstalk between IFNs and Retinoids

Although IFNs and retinoids induce cellular genes using different mechanisms, several studies demonstrated the existence of a crosstalk between their growth-suppressive pathways [95–97]. For example, the PML gene and PML-RAR α are induced by IFNs *via* STAT1-dependent pathway and implicated in the anti-viral actions of IFNs [98,99]. RA-induced expression of *STAT1* augments IFN-induced responses while a mutant STAT1 inhibits RA-induced differentiation in embryonic stem cells [100–103]. Several experimental and clinical studies have shown that IFN/RA synergistically inhibit tumor growth and tumor angiogenesis compared to either agent alone [104–107]. Interestingly, IFN/RA-induced apoptosis occurred in cells that lacked either a functional p53 and/or Caspase-3 implying distinct pathways are employed by IFN/RA. These observations lead us to propose a hypothesis that IFN/RA induces cell death employing novel regulators. To isolate such gene products we employed a genome-wide expression knockout screen and identified the GRIMs (Genes associated with Retinoid-Interferon-induced Mortality). We propose that GRIMs act in early phase of cell death pathways as a cooperative unit that suppresses anti-apoptotic functions and therefore promotes sensitivity of cells to apoptotic assault.

Isolation of the GRIMs

Unlike isolation of genes on the basis of their differential expression, the use of genetic approaches to isolate genes offers a direct path for the identifying critical factors and their mechanisms of action. We have employed an anti-sense technical knockout strategy [108], for isolating genes that regulate cell death in response to IFN/RA treatment [109]. Briefly, total RNA from untreated and IFN/RA-treated cells was pooled to generate a cDNA library. This unamplified cDNA library was cloned into an episomal vector pTKO1 in an anti-sense orientation. Upon transfection of this anti-sense cDNA library and selection with IFN/RA, cells that expressed an anti-sense death-associated gene will selectively survive, while others will not. Episomes from the surviving colonies were rescued by transforming the Hirt DNAs into *E. coli*. Individual episomes rescued in this manner were tested for conferring protection against IFN/RA-induced cell death in a number of cell lines to eliminate false positives. Episomes that consistently conferred protection against IFN/RA-induced cell death were collectively called the GRIMs. Partial sequencing of the inserts revealed that most of these genes were novel. Interestingly, none of the GRIMs was a member of Bcl2, caspase and death receptor families,

consistent with our hypothesis. Isolation of several GRIMs using this approach suggests the existence of a coordinated network of factors that are necessary for executing IFN/RA-induced cell death. Consequently, interference with the expression of even a single GRIM is sufficient to ablate cell death. In this review, we will primarily focus on GRIM-19, which has emerged as a key player in growth control and apoptosis; and therefore, appears to be a new tumor suppressor on the horizon. .

GRIM-19

The GRIM-19 codes for ~16 kDa protein with no apparent sequence-motif or domain identities with other proteins in the public databases. The *GRIM-19* gene maps to a locus on human chromosome 19 p13.2 [110], a region whose loss was associated in prostate cancer progression [111]. The *GRIM19* mRNA is induced by IFN- α/β but not by RA alone. However, the combination of IFN/RA robustly induced gene expression. Maximal *GRIM19* mRNA is induced between 16–48h of IFN/RA treatment in most cells. Immunofluorescence analyses revealed GRIM-19 to be present in the cytoplasm and nucleus as punctate structures indicative of its association with certain protein complexes *in situ* [112]. Indeed, a subsequent study reported GRIM-19 to be a part of purified Complex-I of the mitochondrial electron transport system (ETS) that is linked to ATP generation [113,114]. Cell fractionation studies revealed that GRIM-19 is present in the nuclear, cytoplasmic and mitochondrial fractions (Nallar et al manuscript submitted). Ectopic expression of GRIM-19 induces cell death and moderate levels of it sensitizes cells to IFN/RA-induced apoptosis [112].

Viruses inhibit GRIM-19 for promoting cell survival

The IFN system evolved to inhibit viruses. In turn, some viruses co-evolved mechanisms that combat IFN system by targeting various components of this pathway. Several inhibitors of IFN induction, PKR, STAT1 and STAT2 have been well described in the literature (see refs [33–46] for a review). The initial anti-GRIM-19 activities came from studies on human Herpesvirus-8 (HHV-8) that is associated with Kaposi's sarcoma (KS), B-cell Lymphoma, multicentric Castleman's disease and primary effusion lymphoma in humans [115,116]. The HHV-8 genome, in addition to several structural proteins, also codes for a number of homologues of various cellular proteins *viz.*, viral IL-6 (vIL-6), viral Bcl2 (vBcl2), and three viral homologues of IRFs (vIRFs) *etc* [39,117]. The viral IRFs (vIRFs) are implicated in cellular transformation and also as arsenals to counter the host response against invading pathogen(s) [118,119]. The vIRF1 protein inhibits IFN gene expression by interfering with the interaction of CBP/p300 with co-activator, IRF3 [120–122]. However, it does not bind to DNA, like the mammalian IRFs. We have shown that vIRF1 binds to GRIM-19 and blocks its ability to induce apoptosis [123]. However, vIRF1 also activates the expression of growth-promoting transcription factor c-Myc [124]. The vIRF2 protein inhibits protein kinase R (PKR), leading to a promotion of protein synthesis [118]. The human Papillomavirus (HPV) E6 protein of the high-risk strains, but not the low-risk strains, binds to GRIM-19 [123]. Consistent with these observations, we have found that expression of GRIM-19 is down-regulated in human cervical carcinomas [125]. Other viruses appear to target GRIM-19 *via* different mechanisms. For example, *de-novo* levels of GRIM-19 dropped significantly at 2h after Vaccinia virus infection and maintained at low level as long as 16 hours post-infection [126]. Although the factors/mechanisms involved in this process are not clear, it is likely that cellular apoptosis is ablated to promote viral survival. A more provoking and specific anti-GRIM-19 mechanism is used by human Cytomegalovirus (CMV). This virus produces a highly-abundant non-coding 2.7-kb viral RNA ($\beta 2.7$) that accounts for 20% of total viral transcription [127,128] in infected cells [129]. This RNA enters mitochondrion *via* an unknown mechanism and 'locks-up' GRIM-19 in Complex-I, to render it ineffective at triggering apoptosis [130]. A viral mutant, lacking $\beta 2.7$, was incapable of this activity. The $\beta 2.7$, when expressed from a plasmid was able

to complement the defect of this mutant *in trans*, suggesting its independence from other viral proteins. Although this virus upregulated steady-state levels of mRNAs coding for some subunit proteins in ETS Complexes-I to V, it did not affect *GRIM19* levels [130]. Why and how the virus achieves this balance is worth investigating. Fig. 2 shows some strategies employed by viruses to evade the apoptotic action of GRIM-19.

Cellular targets of GRIM-19 in mediating growth suppression

To gain a better understanding of GRIM-19-induced growth suppression and to know its cellular targets, we had used yeast two-hybrid (Y2H) screens and identified STAT3 as one of its interacting partner [131]. This result was independently corroborated by another study, which employed STAT3 as a bait in a Y2H screen to isolate GRIM-19 [132]. STAT3 is central to several diverse biological functions in mammals including cytokine-driven responses, differentiation, embryonic growth and cell survival [133–135]. In cytokine-regulated pathways and in normal cells, STAT3 activity is tightly regulated by feedback inhibitors. In contrast to this, constitutive activation of STAT3 has been documented in a number of tumors [134, 136–140]. Constitutively-active STAT3 induces the expression of anti-apoptotic members of Bcl-2 family, such as Bcl2, Mcl1 and Bcl-X_L [141,142] and represses the expression of death-receptor Fas [143,144]. A number of oncogenic products such as c-fos, c-myc and cyclin D1 are induced by STAT3 [145,146]. In addition, many advanced tumors produce secretory factors [147] that promote tumor metastasis [148] and inhibit the anti-tumor activity of invading macrophages [149–153]. Interference with STAT3 function promotes apoptosis in several tumor cells [141,142,154–157].

As mentioned earlier, the other known inhibitors of STAT3 activity *i.e.*, PIAS3 and SOCS3 act by preventing its DNA binding activity and blocking JAKs, respectively. The biological impact of PIAS3 on constitutively-active STAT3 is unclear, given most data in a single report was obtained using over-expression strategies [67]. Proteins of this family reported to possess SUMO-like activities [158–161] and act as co-regulators of androgen receptors [162–164]. Their physiologic role in regulating constitutively active or endogenous STAT3 is unclear at this stage. Although SOCS proteins have been shown to inhibit STAT3 activation in the cytokine-induced pathways, oncogene-induced activation of STAT3, which is commonly seen in tumors, is resistant to their inhibitory effects [165]. Interestingly, GRIM-19 interferes with STAT3 in a manner distinct from that of other inhibitors of cytokine-induced JAK-STAT pathways. In response to exogenous and endogenous signals STAT3 undergoes phosphorylation at two critical sites *viz.*, Y705 and S727 [56]. The former is mediated by JAKs and other oncogenic tyrosine kinases to permit dimerization of STAT3 while phosphorylation at S727, although exact kinase that performs this function is uncertain, is absolutely critical for full transcriptional activity of STAT3 [166–169]. Our studies showed that GRIM-19 binds to the trans-activating domain (TAD) of STAT3 and blocks its activity and the S727 is critical for these actions [131]. In contrast, Lufei *et al.*, [132] reported that GRIM-19 binds to the coiled-coil, DNA-binding and linker domains of STAT3. This is surprising given the small size of GRIM-19. The authors did not explain how such a small protein binds to 3 independent domains of STAT3. Because this study mainly used overexpression as a strategy for defining the domain needed for interaction, it is likely to be artifactual association. We used a restoration of mutants into cells lacking STAT3 to define the critical binding regions [131]. However, we have found that TAD alone is insufficient for GRIM-19 binding suggesting an additional site and/or factor(s) mediating this interaction [131]. The failure of TAD alone to bind GRIM-19 may also be due to an improper folding/conformation of when it is expressed separately from the rest of the protein. We have subsequently reported the effects of GRIM-19 binding to a constitutively-active form of Stat3 (S3C) [170]. The S3C protein forms a spontaneous homodimer that mimicks tyrosyl-phosphorylated STAT3 and localizes to the nucleus [145]. Using Western blot analyses, we have shown S3C-GRIM-19 complexes in the nuclear extracts,

which is contrary to the mechanism suggested by Lufei *et al.* Additionally, binding of GRIM-19 to STAT3 or S3C did not prevent their DNA-binding activity as revealed by EMSA and Chromatin Immunoprecipitation analyses of STAT3-responsive gene promoters [131, 152,170], which again contradicted the mechanism suggested by Lufei *et al.* Lastly recombinant GRIM-19 failed to disrupt the DNA binding of STAT3 *in vitro* [131]. It should be noted that Lufei *et al.*'s (2003) conclusions were entirely drawn on the basis of overexpression of STAT3 and GRIM-19 in COS cells. It is likely that overexpression-associated artifacts may explain the hindrance of nuclear translocation of STAT3. Despite these differences with respect to localization of GRIM-19, mechanism of action, intra-cellular environment of complex formation *etc*, both studies agreed on the death-inducing activity of GRIM-19 is mediated, in part, *via* its anti-STAT3 effects. GRIM-19 robustly suppressed S3C-dependent tumor growth *in vivo* [170]. Consistent with its antitumor activities, our recent studies have shown that interference with STAT3 function either by intratumoral administration of shRNAs [171] or GRIM-19 via plasmids [172] caused the regression of pre-established tumor *in vivo*. More importantly, co-expression of shRNA-STAT3 and GRIM-19 via plasmids caused a synergistic suppression of tumor growth. These data demonstrated a direct anti-tumor effect of GRIM-19 in the absence of other proteins.

Our Y2H screens also identified another binding partner HtrA2/Omi, a mitochondrial serine protease, as another cellular target of GRIM-19 [173]. In mammalian cells, inhibitors of apoptosis proteins (IAP) block Caspase activities [174]. One such protein, X-linked IAP (XIAP) inhibits Caspase9 [175]. HtrA2 degrades XIAP to relieve the inhibition on Caspases [176–179]. Our studies identified a functional collaboration between HtrA2 and GRIM-19 leads to a robust degradation of XIAP, leading to a super-induction of apoptosis. The interaction between GRIM-19 and HtrA2 was significantly stronger upon IFN/RA treatment suggesting some post-translational change(s) that enhances their binding [173]. Indeed, a recent study reported dynamic phosphorylation on T113 residue of GRIM-19 in steady-state cells [180]. Interestingly, one of the GRIM-19 point mutations R115P, reported in a case of thyroid cancer [181], is in close vicinity to the above mentioned phosphorylation site. Whether T113 phosphorylation has any biological consequence needs to be experimentally defined. These in conjunction with our earlier finding that the deletion of the C-terminus of GRIM-19 abrogates its death-promoting function [112] appear to be relevant in this context. The binding of GRIM-19 to HtrA2 requires the PDZ domain of the latter protein. This domain is also targeted by other inducers of cell death [182,183].

Another growth-promoting gene product that interacts with GRIM-19 is GW112 [184]. It belongs to the Olfactomedin family of proteins, which are involved in neurogenesis, neural crest formation, dorsal-ventral patterning, cell-cell adhesion, cell cycle regulation and tumorigenesis; and may serve as a modulator of critical signaling pathways, such as those driven by BMP [185]. Importantly, some of these proteins exist as secreted glycoproteins and cell membrane-associated proteins to support cellular signaling [186,187]. GW112/Olfactomedin 4 is highly expressed in the tumors of the upper gastro-intestinal tract. Immunochemical analysis revealed that most of GW112 protein to be in mitochondria and some in the nucleus as distinctive foci. Levels of GW112 reach a peak during early S phase that gradually decline over late S phase. GW112 was required for G₂/M transition [188]. However, reduction of GW112 levels by RNAi resulted only in a slight increase in apoptosis, which is drastically different compared to STAT3 knockdown. Thus, GW112 may exert a STAT3-independent effect on GRIM-19. Overexpression of GW112 attenuated H₂O₂ and IFN/RA-induced apoptosis [188]. Although the exact role of GW112 in growth-promotion is unclear at this stage, three genes products involved in apoptosis and oxidative-stress (PIG12, GADD153 and c-Abl) were down-regulated in cells overexpressing GW112, while the same genes were up-regulated in GRIM-19-overexpressing cells and/or in response to IFN/RA treatment [188]. Interestingly, GW112 is expressed during myeloid lineage development

[189] and binds to cell membrane-associated molecules such as cadherins and lectins [190]. Hence, the exact cellular function of GW112 remains a mystery.

Effect of GRIM-19 on cell cycle

Although GRIM-19 at high levels induces apoptosis, moderate expression of GRIM-19 is tolerated but resulted in growth retardation [112]. We have gained an insight into how GRIM-19 might control this process. Since GRIM-19 is present in multiple cellular compartments and appears to participate in diverse biological responses, we undertook a proteomic analysis of its other binding partners. One of the many proteins identified in this analysis was the cell cycle inhibitor CDKN2A (p16^{Ink4a}). Progression of normal cells from G₁-S-G₂-M-G₀ during division cycle is tightly controlled by the protein kinase activity of a class of serine-threonine kinases called the cyclin-dependent kinases (CDK) [191–193]. As implied in their name, their catalytic activity is dependent on the presence of protein factors known as cyclins [191]. Cyclins, as evident from their name, show phase-specific oscillations in their levels during cell cycle. Overexpression or failure to degrade them leads to oncogenesis [194–196]. In addition to a controlled expression of cyclins (the activators), CDK's activity is blocked by CDK-inhibitors, which determine cyclin-CDK complex formation(s). Two families of inhibitors belonging to Kip/Cip and Ink families regulate CDK activities. The INK4 family includes 4 proteins viz., p15, p16, p18 and p19 [197], which interact with CDK4/6 to suppress cyclin-dependent phosphorylation of the Retinoblastoma (RB) protein [198]. RB, a known tumor suppressor, binds to transcription factor E2F1 and suppresses its ability to drive G₁/S associated gene expression [199]. Phosphorylation of RB by CDK4/6 releases E2F1 from the RB-E2F1 complex, which initiates transcription of proliferation-associated genes, e.g. *CCNB*, *DHFR*, *JUNB* and *TK1* [200,201].

We have found that GRIM-19 specifically interacts with p16 but not with other members of this family. The N-terminus of GRIM-19 was crucial for this interaction while the C-terminal fourth ankyrin-like repeat of p16, which is distinct from the CDK-interacting surface, was necessary for its association with GRIM-19. The GRIM-19/p16 combination caused a synergistic suppression of E2F1-dependent gene expression to cause G₁ arrest. We found that in presence of GRIM-19, p16-bound CDK4 complexes were high thus lowering Cyclin D1-bound CDK4. Interestingly, GRIM-19 alone suppressed E2F1-driven transcription. This could occur *via* two mechanisms: 1) cyclin D1 is direct target of STAT3, whose transcriptional activity is inhibited by GRIM-19; and 2) E2F1 and its dimerizing partner, DP1, are STAT3-responsive genes at transcriptional level [146]. Thus, inhibition of STAT3 by GRIM-19 lowers cyclin D1 levels required for S-phase progression and levels of E2F1 required for S-phase associated gene expression (Sun et al manuscript submitted). Fig.3 schematically represents the effects of GRIM-19 on cell cycle progression.

Tumor suppressor p16 is frequently either mutated or its expression is epigenetically suppressed in a number of tumors [197]. Several studies have shown that mutations or deletions in the second and third ankyrin-like repeats of p16 abolish its anti-CDK4 activity [197]. It was unclear, thus far, how genetic alterations, such as deletions, insertions or point mutations, in the fourth ankyrin-like repeat identified in various types of tumors inactivate p16 [197]. We found that deletion of the fourth ankyrin-like repeat or introduction of clinically-observed mutations into this region ablated the interaction of p16 with GRIM-19 and its ability to synergistically suppress E2F1-responsive gene expression and tumor growth. It appears that GRIM-19 acts a chaperone for p16 in promoting growth arrest in this scenario. Together, these studies identified a novel mechanism of collaboration between two tumor suppressors.

Effects of GRIM-19 on cytoskeleton and cell adhesion

Unlike normal cells, tumor cells grow in an anchorage-independent manner and fail to attach to the substratum as they become metastatic. Activated oncogenes play central roles in these processes by altering transcription of several genes involved in these processes. For example, expression of angiogenesis and metastasis-associated genes are controlled by activated STAT3 in a number of cancer cells [147,172,202–204]. The activated Src oncogene is a major activator of STAT3 [205] and inhibition of STAT3 suppresses Src-induced tumorigenesis [206]. The Src family of tyrosine kinases are important regulators of various cellular responses *viz.*, cellular transformation, altering transcription, and cytoskeletal properties [207–211]. Based on these observations we investigated if GRIM-19 could overcome Src-induced oncogenesis. Indeed, GRIM-19 suppressed Src-induced cellular transformation *in vitro* and *in vivo* by down-regulating the expression of a number of STAT3-responsive genes [212]. In addition, GRIM-19 inhibited Src-induced cell motility. Interestingly, Src-induced changes in adhesion were suppressed by GRIM-19. Src induced tyrosyl phosphorylation of focal adhesion kinase, paxillin, E-cadherin and γ -catenin, proteins involved in cell adhesion [207–211]. Tyrosyl phosphorylation of these proteins disrupts cellular adhesion. While Src was able to induce phosphorylation of these proteins in transformed cells, these effects were lowered by GRIM-19 [212]. The specificity of this was further shown by a reversal of GRIM-19 effects on Src-induced cellular transformation by a GRIM-19-specific shRNA. GRIM-19-mediated inhibition of Src-induced tyrosyl phosphorylation of cellular proteins, such as FAK and paxillin, seemed to occur independently of the STAT3 protein, given an equivalent effect of GRIM-19 on Src-induced transformation after knocking down STAT3. As said earlier, Src stimulates cell motility. Expression of v-Src, controls several cellular activities including cell morphology, adhesion, motility, invasion *etc* [213]. By associating with cytoskeleton, v-Src causes changes in cell morphology and structure. For example, cells transformed by v-Src convert from a well-spread to an extremely rounded shape, a characteristic of high motility and invasiveness. Most of these changes were associated with a reorganization of the cytoskeleton, including loss of stress fibers, adhesion plaques and formation of podosomes (dot-like bundled F-actin-rich structures) [214]. One of the important substrates of v-Src is cortactin, a filamentous actin-bundling protein [215] which serves as a scaffold linking the v-Src signaling pathway to the organization of cytoskeleton. Tyrosyl phosphorylation of cortactin by v-Src occurs in a progressive manner [216]. Studies have shown that phosphorylation of cortactin by v-Src reduces the affinity of Cortactin for actin and its ability to stabilize cross-linked actin filaments [217]. Depletion of Cortactin lead to a specific loss of podosomes and re-expression of cortactin mutants lacking phospho-accepting residues (Y⁴²¹, Y⁴⁶⁶ and Y⁴⁸²) did not restore podosome formation [218]. GRIM-19 blocked v-Src-induced cell motility by inhibiting the tyrosyl phosphorylation of Cortactin. This could be one of the mechanisms that explain the suppressive role of GRIM-19 on v-Src-induced podosome formation. The N-terminus of GRIM-19 was required for exerting these effects. More importantly, a clinically-observed mutation in this region significantly disrupted the inhibitory effect of GRIM-19 on v-Src-induced cell motility [219]. Consistent with these observations, deletion of N-terminus resulted in a loss of the anti-metastatic activity of GRIM-19 *in vivo*. GRIM-19 had no significant effect on the cellular transformation induced by other oncogenes such as *myc* and *Ha-ras* [212]. In summary, GRIM-19 not only blocked Src-induced gene expression through STAT3 but also the activation of cell adhesion and motility-associated proteins. Similar effect on cell motility is controlled by another protein Stathmin that destabilizes microtubules [220]. STAT3 interacted with Stathmin thus antagonizing its microtubule-destabilizing activity. The effect of GRIM-19 in this process is unclear. Since GRIM-19 interacts with STAT3, it is tempting to speculate that these inhibitory networks may be operative as well.

Mitochondrial electron transport and GRIM-19

Mitochondria utilize a coupled network of membrane-anchored multi-protein complexes to generate reductive power, and ATP, required for driving many cellular functions. Generally referred to as the OXPHOS system, it is composed of five structurally and functionally distinct protein complexes comprising of at least 88 different proteins, of which 75 are coded by the nuclear genome while 13 are coded by the mitochondrial DNA. However, the minimum number of proteins required to form a functional complex, in each of these complexes, is uncertain in higher eukaryotes, which is in contrast to the simpler bacterial systems that show conservation of some of the subunit proteins and yet are able to function normally. Thus, OXPHOS system in higher eukaryotes appears to house many proteins whose role(s) are unclear. Of the five major complexes, Complex-I (CI or RC1) is the biggest comprising of 45 distinct proteins, seven of which are coded by mitochondrial DNA in bovines (Carroll et al., 2006). Subsequent to our identification of GRIM-19, Fearnley et al [113,114] demonstrated GRIM-19 to be a part of CI in bovine heart in line with our report that its level was high in skeletal and cardiac muscle [112]. Later, it was demonstrated that homozygous deletion of *Grim19* caused embryonic lethality [221] and thus essential for early embryonic development in mouse and to be a functional component of CI using *in vitro* studies. Even though the authors claimed absence of GRIM-19 affected other complexes in the mitochondria, evidence presented was inconsistent to such a claim. However, subunit-dependent regulation in assembly has been reported using other OXPHOS-associated proteins such as AIF, NDUFB6, NDUFS7 and NDUF9, all of which are part of CI [222] (Vahsen et al., 2004). Interestingly, the same group had observed STAT3-GRIM-19 co-localization that were non-mitochondrial and mitochondrial (Lufei et al., 2003). Furthermore, the use of truncations, deletions and dominant-negative constructs of GRIM-19 has not addressed its role in CI [221,223]. However, these authors acknowledge that differentially modified and/or isoforms GRIM-19 forms can be non-mitochondrial with novel function(s) that agrees with rotenone-induced mitochondrial stress promotes relocalization of GRIM-19 within the cell (Reeves et al., 2007).

Recently, a role for Janus tyrosine kinase, Tyk2, in mitochondrial respiration in mouse primary pro-B lymphocytes was reported (Potla et al., 2006). Although Tyk2^{-/-} mice were phenotypically normal, cells from these mice exhibited abnormal responses to inflammatory challenges in different cell types; specifically pro-B cells were markedly deficient in basal oxygen consumption and exhibited a significant decrease in steady-state ATP levels which were attributed to impaired complex-I, complex-III and complex-IV functions of the mitochondrial ETS. However, Tyk2 was required for maintenance of both complex-I-dependent respiration as well as induction of apoptosis in cells incubated with IFN- β . Consistent with the role of Tyk2 in the regulation of tyrosyl phosphorylation of STAT3, expression of S3C could restore mitochondrial respiration and IFN- β -stimulated apoptosis in Tyk2^{-/-} cells. Though S3C did restore IFN- β -stimulated apoptosis, it was incomplete suggesting another Tyk2-dependent pathway is necessary for the full response. This appears to be an enigma wherein STAT3 phosphorylation stabilizes complex-I respiration, Tyk2 and tyrosyl-phosphorylated STAT3 are required for apoptosis, the same JAK-STAT pathway induces GRIM-19 while the receptor stimulation causes GRIM-19 release from complex-I. It is debatable as to which route of signaling is stronger to achieve the outcome *i.e.*, survival / apoptosis. These results could also be interpreted as a novel mechanism to control STAT3 activity wherein, some GRIM-19 from complex-I gets released to bind STAT3 and control the temporal and/or spatial activity of STAT3 in the nucleus. However, novel roles for non-tyrosyl phosphorylated STAT3 have been reported [146] suggesting other factors and/or promoter-context may also be important. More importantly, the generality of this observation to other cell types, other than pro-B cells, needs to be verified.

GRIM-19 in intracellular bacterial defense

Barnich *et al.*, [224] identified that GRIM-19 binds to NOD2 (nucleotide-binding oligomerization domain containing 2) using Y2H screen. NOD proteins are a group of intracellular bacterial molecular pattern-recognition sensors that are thought to utilize RIP2/RICK and induce NF- κ B activation [225,226]. Together with cell membrane-associated TLRs (Toll-Like Receptors), NOD proteins play important roles in innate and acquired immunity. However, Barnich *et al.*, [224] found GRIM-19 is crucial for NF- κ B activation following NOD2-mediated recognition of bacterial muramyl dipeptide. GRIM-19 appeared to be a key component in NOD2-mediated innate mucosal responses. Interestingly, GRIM-19 localized in vesicles, which are not near the cell membrane, not in mitochondria or the nucleus in this study whose significance needs to be addressed. Barnich *et al.*, [224] also reported up-regulation of GRIM-19 when challenged by intra-cellular bacteria.

Intracellular bacteria as inducers of GRIM-19

As mentioned above, coupling of TLR4 and NOD2 signals is necessary for upregulating innate immune responses. It is unclear whether activation of cell membrane-associated TLRs results in an up-regulation of GRIM-19. Infection with an intracellular bacterium *Salmonella typhimurium* for 2h caused a 2.26-fold rise in *GRIM19* mRNA levels, which was not observed with *E. coli* infection [224], suggesting that intracellular bacteria were capable of inducing GRIM-19. Indeed, BCG-CWS upregulated GRIM-19 levels in monocytes and dendritic cells prepared from peripheral blood [227]. Whereas only a live periodontal pathogen (*Porphyromonas gingivalis*) up-regulated GRIM-19 levels in peripheral blood monocytes, neither its LPS nor its fimbrial proteins could induce *GRIM19* [228]. The kinetics of GRIM-19 upregulation in CaCo2 cells is very rapid compared to stimulation by BCG-CWS or *P. gingivalis* or IFN/RA. Eventhough this difference could be a cell-specific effect, the mechanism and/or pathway(s) involved in it merits investigation. These results suggest that 1) entry of pathogen via natural route, can up-regulate apoptotic machinery very rapidly to control the infection; 2) cells of the immune system have some time-lag in up-regulating GRIM-19; and 3) an initial signal from bacteria might induced GRIM-19 through a secondary mechanism. In an analogous system, differential response and enrichment of GRIM-19 ESTs was observed in hemocytes from mosquitoes, *Armigeres subalbatus* and *Aedes aegypti*, when challenged with *E. coli* and *Micrococcus luteus* [229]. The latter is an intracellular bacterium. These results are also consistent with our recent observations where intracellular bacteria were able to exert a strong anti-tumor response, against a tumor that expressed constitutively active STAT3 [171]. This appears to occur through an induction of GRIM-19.

Patho-physiological relevance of GRIM-19

Although we originally described GRIM-19 as a growth-regulatory protein involved in IFN/RA-induced pathways, recent studies showed its involvement in a number of different pathogenic processes. Chronic Obstructive Pulmonary Disease (COPD) is a complex disease involving two distinct lung pathologies: emphysema and chronic bronchitis, caused by cigarette smoking [230]. It accounts for 80–90% of COPD cases in the United States [231]. However, only 15–20% of heavy smokers develop clinically-significant airflow obstruction, suggesting a genotypic influence in the development of COPD. Genes that determine susceptibility to COPD progression are poorly understood [232]. Studies using SAGE, Microarrays and qRT-PCR, [233] showed that *GRIM19* mRNA levels were higher in smoker's lung compared to non-smokers. However, mitochondrial ETS complex-I activity was reduced by 50% in these patients.

Apoptosis-inducing factor (AIF) is a mitochondrial flavoprotein which translocates to the nucleus where it participates in apoptotic chromatinolysis [234–236]. The physiological role

of AIF in the mitochondria is not clear at this stage. Though AIF is not a part of sub-complexes of CI or mature CI or any super-complexes, it is required for proper assembly and/or maintenance of CI. AIF knockdown resulted in decreased GRIM-19 (NDUFA13), NDUFB6, NDUFS7 and NDUFA9 levels, all being part of CI [222]. However, AIF^{-/-} cells still had complete CI but in reduced abundance resulting in lower enzymatic activities and metabolized glucose like cancer cells.

In a study of patients with inflammatory bowel disease *viz.*, Crohn disease and Ulcerative Colitis, Barnich *et al* [224] showed a significantly lower abundance of GRIM-19 in the involved areas compared to the non-involved areas of the intestinal mucosa. The mechanism(s) of such down-regulation is/are not known. It is likely that chronic inflammation in these diseases may permit a neoplastic growth in the absence of GRIM-19. This may be a good working model for accessing the role of GRIM-19 in the development of neoplasia and metastasis.

In a recent collaborative study we have shown that GRIM-19 levels are significantly upregulated in the affected areas of rat brain following focal cerebral ischemia. Upon treatment of neuroblastoma cell line with IFN/RA for 48h, GRIM-19 levels were up by ~15-fold, while focal ischemia for 24h could up-regulate *GRIM19* mRNA ~32-fold but only 50% increase in protein level [237]. This discordance between protein and RNA levels could be due to an inhibition of protein synthesis in ischemically-damaged cells. However, a cell-specific effect was also evident with respect to *GRIM19* induction by hypoxia; the ipsilateral lobes had higher GRIM-19 levels compared to the contralateral lobes and the neurons had higher GRIM-19 levels compared to astrocytes. Based on these and the fact that GRIM-19 is induced in bacterially-infected cells; we speculate that GRIM-19 may have an unspecified role in cellular stress responses. These issues need to be addressed in the future.

GRIM-19 and Cancer

The full development of human cancer involves multiple genetic changes. Mutations and/or repression and/or loss of genes coding for transcription factors and apoptotic machinery have been implicated in tumor growth [238,239]. As mentioned earlier, viral and cellular oncogenic proteins bind to GRIM-19 and inhibit its death-activating function. Indeed, mutations in human *GRIM19* were reported in Hürthle cell thyroid carcinomas [181]. To gain a better understanding of molecular changes that appear in primary renal cell carcinoma (RCC), we compared the proteomes of isogenic normal kidney and RCC [240]. Results from this study revealed that: 1) 93% of RCCs lost GRIM-19 expression while the remainder had weaker expression, (2) Loss of GRIM-19 appears to be a characteristic of clear cell type RCCs and (3) a low frequency loss of GRIM-19 also occurs in transition cell carcinomas. In most cases, tumor-derived *GRIM19* mRNA seems to harbor no mutations, except for a conservative substitution in one case [240]. In those cases where complete loss of mRNA occurred, no gross deletions in *GRIM19* gene were observed as analyzed by genomic PCR of tumor and normal DNAs (Nallar and Kalvakolanu, unpublished observations). Methylation and/or loss of transcription factor engagement with the promoter could be the reason for loss of expression. More importantly, loss of GRIM-19 associated with a robust upregulation of STAT3 and STAT3-responsive genes in these tumors (Fig. 4).

Our studies with primary human prostate carcinomas showed loss of GRIM-19 correlated with high STAT3 expression and upregulation of several cell growth and metastasis-associated genes [172]. Indeed, restoration of GRIM-19 suppressed the expression of such genes and suppressed growth *via* apoptosis. In primary cervical carcinomas loss of GRIM-19 resulted in hyper-induction of STAT3-responsive genes. Restoration of GRIM-19 caused a robust tumor suppression which was associated with a loss of tumor angiogenesis and expression of matrix metalloproteinases [125].

We have also profiled a few oral squamous cell carcinoma (SCC) samples where the levels of GRIM-19 was not significantly different, except for one sample that had a complete loss of expression, but STAT3 levels were significantly higher in the tumors of matched samples (Nallar and Kalvakolanu, unpublished). It appears that mutations are present in *GRIM19* from these tumors. We are currently investigating the biological importance of these mutations. An earlier study by Nindl *et al.*, (2006) [241] on cutaneous SCC, by microarray expression profiling, concluded no significant differences with respect to *GRIM19* levels. A recent study by Gong *et al.*, (2007) [242] reported higher levels of STAT3 and lower levels of GRIM-19 in colorectal carcinomas compared to control samples. No mutation was detected in *GRIM19* from the 23 specimens studied. Together these studies indicate a major role for GRIM-19 in tumor suppression.

Conclusions

Since their discovery about 50 years ago, IFNs have been on the forefront of basic and clinical investigations and have emerged as potent therapeutic agents for certain neoplasias and neuro-degenerative diseases. Although a number of studies identified 100s of IFN-regulated genes, a small portion of these genes, >10–15 %, are fully characterized. The antiviral action of IFN is slightly well characterized. However, IFN-regulated cell growth control beyond JAK-STAT pathways is poorly defined. In this review we described the actions of a novel IFN/RA-induced gene, *GRIM19*, which appears to be potential tumor suppressor. Although GRIMs were appears to be IFN/RA-induced cell death regulators *prima facie*, they may participate in growth-regulatory processes controlled by other ligands and in other physiologic processes in normal cells. Indeed, recent studies have shown a) GRIM-19 to be a part of the membrane module of CI, that is added during the pre-final stage of CI assembly [243]; b) GRIM-19 is essential for Ca^{++} homeostasis during embryogenesis [244] and c) GRIM-19 is upregulated during ischemia. That said, the role of mitochondrial GRIM-19 is unclear, given many organisms that lack this subunit still produce normal levels of ATP. Fig.5 represents our current understanding of GRIM-19-regulated anti-tumor actions. Recent studies showed a mitochondrial role for STAT3 in maintaining CI [245]. Given GRIM-19 is an inducer of apoptosis and an inhibitor of STAT3, in steady state both proteins counter balance their effects. Under conditions favorable for apoptosis, GRIM-19 associates with HrA2 and aids in the destruction of XIAP. Nuclear GRIM-19 lowers the levels of anti-apoptotic proteins like Bcl-2 and Bcl-X_L to promote mitochondrial damage, leading to full-blown apoptosis.

The maintenance of genomic integrity is critical for preventing tumor development. Cell cycle checkpoints play a crucial role in these processes. The dominant G₁ & G₁-S checkpoint responses are routed through p53 and RB, which are most commonly deregulated in human cancers [246–248]. The transcription factors E2F1 and c-myc, targets of unphosphorylated STAT3, regulate the expression of genes associated with cell cycle progression such as Cyclins D1 and E that control phase transition [249,250]. The ability of GRIM-19 to attenuate STAT3 responses adds a new dimension to this checkpoint. More importantly, GRIM-19 seems to integrate well into RB arm of cell cycle check point by virtue of its ability to associate with p16 to enforce a robust inhibition of CDK activity and E2F1-regulated gene expression. Like p53 and RB, human tumors show loss of expression and/or mutations in *GRIM19*. In this regard, GRIM-19 may now be classified as a new tumor suppressor, given a loss of its expression in tumors and accumulation of mutations in certain tumors. Furthermore, GRIM-19 associates with p16^{Ink4a} to enforce a robust inhibition of cell cycle progression. In S phase, GRIM-19 appears to block GW112, whose function is unclear at present. The G₂ and G₂-M checkpoint is controlled by the activity of Cyclin B1/CDK1 [251,252]. Incidentally, Cyclin B1 also happens to be a STAT3-responsive gene that is inhibited by GRIM-19. Thus, GRIM-19 may act as a ‘brake’ throughout the cell-cycle in a normal cells emphasizing its role as a wide-spectrum growth controller *i.e.*, in the absence of any stress-like situation(s). Like p53 and RB,

GRIM-19 is targeted to inhibition by viral proteins (Fig. 1). That said, cooperativity among GRIMs is required for full-blown apoptosis upon IFN/RA treatment that is distinct from known pathways of apoptosis and the GRIM machinery even causes cell death in p53 and Caspase3-deficient cells [253]. Unlike most apoptosis-inducing proteins, whose cellular localizations are delineated, GRIM-19 appears to move around inside the cell (Fig. 2) like a sentinel to control functions *viz.*, apoptosis, cell cycle, innate immunity, cell motility, and CI assembly *etc.* Does the structure of GRIM-19 permit such a diverse function? Interestingly, GRIM-19 shows no conserved domain structures and/or motifs. Database searches using PSI/PHI-BLAST revealed the N-terminal stretch of amino acids have good similarity to influenza Hemagglutinins and capsid protein of echovirus V. We have also used structure prediction tools to model 3-D structure of GRIM-19, which revealed an ultra-compacted central and distal α -helical region was preceded by a free N-terminal region. Consistent with this model, mutation and/or deletion of amino acids (1–17) of the N-terminus of GRIM-19 resulted in a loss of its inhibitory effect on v-Src and CDK4. Thus, different regions may be required for specific functions of GRIM-19. However, it is possible that GRIM-19's activity is additionally controlled by post-translational modifications, which permit its localization, interaction and regulation of cellular responses. Although one event of phosphorylation has been reported in GRIM-19 [180], its significance is unclear. Given the kinetics of GRIM-19 regulation, the stress factors that activate the apoptotic behavior of GRIM-19 are currently being investigated.

Abbreviations used

Bcl-2	B-cell lymphoma-2
BH	Bcl2 homology
FADD	Fas-associated protein with a death domain
GRIM	Gene associated with Retinoid-Interferon-induced Mortality
HtrA	High temperature resistance locus A
IFN	Interferon
ISG	Interferon-stimulated gene
ROS	Reactive oxygen species
STAT	Signal transducer and activator of transcription
RB	Retinoblastoma susceptibility protein
CDK	cyclin-dependent protein kinase
XIAP	X-linked inhibitor of apoptosis

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Fig. 1.

Major Apoptotic pathways. The extrinsic pathway is driven by Fas-type death receptors. Upon engagement with their ligands, these receptors trimerize and the death adaptor protein FADD is recruited to the intracellular death domain of Fas. FADD then recruits pro-caspase-8 into this complex completing the formation of the death-inducing signaling complex (DISC). The induced juxtaposition of pro-caspase-8 results in autocatalytic cleavage and activation of caspase-8. Caspase-8 then dissociates from the receptor and enters the cytoplasm to initiate the cleavage other caspases or cellular proteins. The intrinsic pathway (activated by cytotoxic drugs, radiation or reactive oxygen species) involves the damage of mitochondrion. The Bcl2 and Bax proteins counteract each other in steady state. Signal-induced rise in Bax and/or dissociation of Bcl2:Bax complex by Bak results in the formation of Bax:Bax or Bax:Bak oligomers, which insert into the mitochondrial membrane and open permeability transition pores (PTP). This leads to the release of many apoptotic inducing factors such as AIF, HtrA2, Endonuclease G (EndoG), Smac/Diablo and cytochrome-c. Cytochrome-c associates with apoptosis-activating factor-1 (APAF1) and pro-caspase-9 leading to formation of 'Apoptosome'. Activated caspase-9 from this complex activates other caspases, which dismantle the cellular proteins required for viability. Although thought to operate independently, in some cell types, the intrinsic and extrinsic pathways cross regulate each other. In these scenarios, caspase-8 from the DISC cleaves the pro-apoptotic Bcl2-like protein, Bid, to generate a truncated version, t-Bid. The latter causes mitochondrial damage, which amplifies the apoptotic response through the formation of Apoptosome. A number of proteins in this pathway are controlled by IFNs and other cytokines.

Fig. 2. Viral interference with GRIM-19. Various mechanisms that suppress GRIM-19 have been indicated.

Fig. 3. GRIM-19 controls multiples nodes on cell cycle. Various phases of cell cycle are indicated. Growth factor-induced synthesis and association of cyclin D with CDK4/6 stimulates the kinase activity of CDK, which then phosphorylates tumor suppressor RB. Phosphorylation of RB and of E2F1 dissociates the complex. E2F1 is a transcription factor. It forms a heterodimer with its partner, DP1, and binds to the promoters of the genes critical for S phase. CDK4/6 activity is inhibited by p16. The p16/GRIM-19 complex promotes a robust suppression of CDK activity. As a result RB phosphorylation is suppressed. Failure to release E2F1 from the RB/E2F1 complex prevents its transcriptional activity. GW112/Olfactomedin 4 promotes G2/M transition. Inhibition of this protein by GRIM-19 leads probably a failure to mitotic division. As a result cells might decide to enter into apoptosis. Note: Although both processes have been drawn in this figure only one effect of GRIM-19 may operate depending on the cell type and growth inhibitory signal. Green pointed arrows indicate a positive effect. Blunt red arrows indicate a negative effect.

Fig. 4.

Loss of GRIM-19 upregulates STAT3-responsive gene expression. Panels A–D show relative levels of STAT3-responsive genes levels by real-time PCR analyses. Isogenic pairs of primary normal (N) clear cell type RCC (T) from 3 separate patients are shown. All data were normalized to that of ribosomal protein L32. Panel E shows Western blot profile of STAT3, GRIM-19 and Actin expression levels in these samples. Note the loss of GRIM-19 in tumors, which correlates with high STAT3 levels.

Fig. 5.

The anti-tumor mechanisms of GRIM-19 action. It targets STAT3 and Src to promote pro-apoptotic gene expression and induction of apoptosis. By associating with HtrA2, it augments the proteolytic cleavage of XIAP, which promotes caspase activity. By inhibiting Src-induced tyrosyl phosphorylation of adherence and cell motility-associated proteins like Cortactin GRIM-19 prevents the metastatic behavior of tumor cells, independently of STAT3. By associating with p16, GRIM-19 inhibits cell cycle progression by enforcing the inhibition of CDK4/6. GRIM-19 can perform these functions only when it is present in more than one intracellular compartment. GFR: growth factor receptors. Red colored-lines and arrows represent GRIM-19-dependent inhibitory and stimulatory pathways, respectively. Blunted and sharp arrows indicate negative and positive regulation of the indicated step.