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GRIM-19: A master regulator of cytokine induced tumor suppression, metastasis and energy metabolism

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Abstract

Cytokines induce cell proliferation or growth suppression depending on the context. It is increasingly becoming clear that success of standard radiotherapy and/or chemotherapeutics to eradicate solid tumors is dependent on IFN signaling. In this review we discuss the molecular mechanisms of tumor growth suppression by a gene product isolated in our laboratory using a genome-wide expression knock-down strategy. Gene associated with retinoid-IFN-induced mortality –19 (GRIM-19) functions as non-canonical tumor suppressor by antagonizing oncoproteins. As a component of mitochondrial respiratory chain, GRIM-19 influences the degree of “Warburg effect” in cancer cells as many advanced and/or aggressive tumors show severely down-regulated GRIM-19 levels. In addition, GRIM-19 appears to regulate innate and acquired immune responses in mouse models. Thus, GRIM-19 is positioned at nodes that favor cell protection and/or prevent aberrant cell growth.

Keywords

cytokines; anti-tumor action; glucose metabolism; cell motility

Introduction

A fundamental requirement for multicellular organisms is to maintain healthy tissue architecture by removing excessive unwanted cells or damaged/infected cells, and replenishing them with a new set. Damage emerges from external sources like pathogens, non-biological agents or internal sources such as changes in the levels of cytokines, growth and survival factors synthesized by the host or oxidative damage associated with metabolism. Any dysregulation of these processes results in various pathologies, including the development and progression of cancer. Development of neoplastic cells is held in check *via* 4 major mechanisms: 1) senescence, 2) cell cycle arrest, 3) activation of apoptosis, and 4) immune responses. Cytokines play critical roles in these processes [1]. Cytokines are both secreted and membrane bound proteins, important for cell-to-cell signaling. This review will

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address how disruptions in cytokine-induced signaling pathways can lead to acquisition and maintenance of sustained proliferative capacity and loss of growth-inhibitory mechanisms. Primarily, we focus on the actions of a novel tumor suppressor, Gene associated with Retinoid-Interferon-induced Mortality-19 (GRIM-19) [2].

The IFN family of cytokines is constituted by multiple sub-type proteins, α , β , γ , δ , ϵ , λ , τ , ω , and δ . Classically IFNs were described as agents that establish an anti-viral state in cells by inducing the expression of cellular IFN-stimulated genes (ISGs). It is now clear that IFNs inhibit cancer cell growth and proliferation either by direct induction of anti-cellular gene expression or by promoting immune response. These two modes of anti-tumor effects, however, are not mutually exclusive. IFNs are grossly classified into three types, based on their usage of cell surface receptors (see [3] for a review). Type-I IFNs (IFN- $\alpha/\beta/\epsilon/\kappa/\omega$) signal through a common heterodimeric receptor constituted by the IFNAR1/2 subunits, type-II IFN (IFN- γ) signals through a different heterodimeric receptor (IFNGR1/2), while type-III IFNs (IFN- λ 1/2/3) signal through a heterodimeric receptor consisting of IFNLR1 and IL-10RB subunits [4]. IFNLR1 and IL-10RB subunits are also used by IL-28 and IL-10, respectively. IFNs primarily regulate immune response through the Janus tyrosine kinase (JAK) and Signal transducer and activator of transcription (STAT) pathways. IFNs act as sentinels to prevent and/or eliminate tumor development [1, 5]. Administration of type-I IFNs into tumor-bearing animals inhibited tumor growth in pre-clinical studies [6]. IFNs inhibit tumor growth as efficiently as many clinically used therapeutics [7]. However, the therapeutic utility of IFNs for human cancers is limited by their debilitating side effects and tumor stage-specific differences in gene expression programs of cancer cells [8]. Nonetheless, the single-agent efficacy of IFNs is comparable to many currently used chemotherapeutics. A number of recent reports indicate that the success of conventional chemotherapeutics, targeted anti-cancer agents, radiotherapy and immunotherapy relies on type-I IFN signaling [9–14] *in vivo*. As a result, several human cancers accumulate IFN signaling defects to escape from growth suppression [15]. Several experimental [16, 17] and clinical studies [18–20] showed a therapeutic benefit of combining IFNs with other agents. One such combination is the use of vitamin-A metabolite, *all-trans* retinoic acid (ATRA or RA), which is known to cause cell differentiation. RA itself exhibits significant anti-tumor effects in head and neck cancers [21] and acute promyelocytic leukemia [22]. Although IFNs and RA induce growth suppression using different mechanisms i.e., gene products, pre-treatment with RA followed by IFN only showed promising tumor suppression [6]. The mechanistic bases for this cross-talk can be found in our earlier publications [23, 24]. In this review we will focus on an IFN/RA-inducible gene product, GRIM-19, that emerged as a tumor suppressor over the years.

The GRIMs

Although it was clear that IFN and retinoid combinations exert potent tumor-suppressive effects, the molecular bases for this effect were not known. IFN/RA combination was known to induce apoptosis in cells that lacked functional p53 and/or caspase-3 proteins. Therefore, we hypothesized that the anti-tumor effects of IFN/RA combination are mediated by novel factors. To identify these, our lab employed a genome-wide expression knockdown strategy [25] that permitted the isolation of cell-death associated genes. This approach does not

require *a priori* knowledge of the gene products involved. Briefly, a total cDNA library was expressed in an anti-sense orientation under the control of an IFN-responsive promoter from an episomal vector and the transfected cells were stimulated with IFN/RA. The use of an IFN-responsive promoter for anti-sense expression in this strategy mandates the functioning of JAK-STAT signaling. As a result, this strategy does not permit a general resistance to IFNs through a knockdown of the receptor or the signaling components by anti-sense transcripts. Upon treatment, cells harboring the potential mediators of IFN/RA-induced cell death will survive. The surviving cell clones were expanded and the isolated episomes [26] were sequenced to identify the gene products. Based on this observation, they were named as Gene-associated with Retinoid-Interferon induced Mortality (GRIM). Interestingly, none of the isolated genes in this screen were known apoptosis inducers like caspases, pro-apoptotic Bcl2 and/or death receptor family members. Twelve distinct GRIMs were isolated and research has been performed on three genes *viz.*, *GRIM-12* (HGCN symbol: *TXNRD1*) [27], *GRIM-19* (HGCN symbol: *NDUFA13*) [2] and *GRIM-1* (HGCN symbol: *SHQ1*) [28]. GRIM-12, a thioredoxin reductase, protects cells from oxidative stress while GRIM-1 regulates RNA metabolism. This review will focus on the biological actions, physiological and pathological relevance of GRIM-19.

GRIM-19

The gene for human *GRIM-19* is located on chromosome 19p13.2 [29], which is syntenic to mouse 8C1. Both human and mouse *GRIM-19* genes contain 5 exons with one major exception - the first exon of mouse *Grim-19* gene is a long 5' UTR. In all human cells examined to date, an abundant *GRIM-19* transcript (~550 nt) that codes for a 144-aa protein with a M_r of 16 kDa. The mouse *Grim-19* mRNA (~1200 nt) still codes only for a 144-aa protein. The GRIM-19 protein has no apparent homology to other proteins within their respective genomes. The gene for *GRIM-19* is highly conserved in eukaryotes in spite of the differences observed across phyla. Subsequent to our report on the discovery GRIM-19, a mass spectrometric analysis of protein components of the purified bovine heart mitochondrial electron transport chain (ETC) complex revealed the presence of GRIM-19 in respiratory complex-1 (RC-1) commonly known as NADH dehydrogenase (ubiquinone) [30]. This was consistent with our immunofluorescence studies, which revealed the presence of GRIM-19 protein in mitochondria [2]. Apart from this, however, a significant fraction of GRIM-19 protein is present in the nucleus and cytoplasm (non-mitochondrial fractions) whose exact function(s) is not known. The following sections summarize recently described functional interactions of GRIM-19. For details on previously described functions of GRIM-19, the reader is suggested to read our earlier reviews [24, 31].

Non-mitochondrial functions of GRIM-19

Using yeast two-hybrid screens, we and others have identified several proteins that interact with GRIM-19. While our studies were focused on identifying GRIM-19-interacting proteins, others have identified GRIM-19 as a binding partner for their protein(s)-of-interest. The first mammalian protein reported to interact with GRIM-19 was STAT3 [32, 33]. OLFM4 (GW112) [34], HTRA2 [35] and NOD2 [36] were identified later by this approach, while CDKN2A (p16^{INK4A}) was identified in a proteomic analysis [37].

Effects on STAT3

Following cytokine-receptor engagement multiple intra-cellular signaling pathways are rapidly activated leading to the induction of several cellular genes. Among these, the JAK-STAT signaling pathway is by far the best understood. In these pathways, non-receptor tyrosine kinases (NTKs) of the Janus kinase (JAK) family associate with the intracellular surfaces of cytokine receptor, whose activity is stimulated following a ligand-induced aggregation of receptor. Following this, receptor is tyrosine phosphorylated providing an anchoring point for the Signal Transducer and Activator of Transcription (STAT) proteins, which allows their activation by JAKs. STAT proteins act as both signal transducers and activators of transcription. Four distinct JAKs are known to activate seven STAT proteins by phosphorylation, depending on the cytokine (see [38] for a review). In the steady state, STAT proteins exist as latent cytoplasmic proteins. JAK-induced phosphorylation of STAT at a critical tyrosine residue allows them to form dimers with their SH2 domains. Receptor tyrosine kinases (RTKs), like growth factor receptors, and other NTKs belonging to the Src-family of kinases (SFKs) also activate some STAT proteins by a similar mechanism but independently of the JAKs [39]. Among the seven family members, STATs 1 and 3 are activated by a number of cytokines. STAT3 is unique among STATs, as it is activated by RTKs (cytokine and growth factor) as well as NTKs (JAKs and SFKs). Hence, STAT3 regulates several biological functions in mammals including embryonic development [40], cell survival by inhibiting apoptosis [41], growth factor and cytokine-driven responses [38], liver function [42] etc (see Yu et al [43] for a recent review on STAT3 signaling in cancer). Phosphorylation of the Y⁷⁰⁵ residue of STAT3 occurs after ligand binding to the receptor, leading to its dimerization and rapid entry into the nucleus. Phosphorylation of the S⁷²⁷ in the trans-activation domain (TAD) is also critical for the full spectrum of STAT3 transcriptional activity [44]. STAT3 activity is maximal when both Y⁷⁰⁵ and S⁷²⁷ residues are phosphorylated [45]. One report suggested that GRIM-19 interaction with the non-TAD portion of STAT3 protein prevented its nuclear entry [32]. In contrast to this, we have found that GRIM-19 binding to STAT3 requires the S⁷²⁷ residue and the TAD [33]. GRIM-19, without preventing nuclear entry or DNA binding of STAT3 inhibited transcription (Fig. 1). Support for this mechanism comes when GRIM-19 inhibited a spontaneously-dimerizing Stat3 (S3C)-driven transcription of *Bcl2* gene [46]. Using ChIP assays, we have demonstrated the physical presence of GRIM-19 at a STAT3-binding motif of this gene promoter. Such promoter binding of GRIM-19 required STAT3. Intracellular signals like redox imbalance and reactive oxygen species (ROS) regulate Bcl-2 family of proteins either to protect or destroy a cell [47, 48]. These proteins localize to the outer mitochondrial membrane (OMM) where they regulate mitochondrial membrane permeability and hence the release of cytochrome C from the inter-membrane space (IMS). Cytochrome C in the cytoplasm triggers the formation of 'Apoptosome' and initiates cell death. The Bcl-2 family of proteins functions as homo and/or hetero dimers. Anti-apoptotic Bcl-2 members are upregulated in tumors that contributes to apoptosis resistance while depletion of Mcl1, an anti-apoptotic member, resulted in senescence [49]. Thus, GRIM-19 can reduce Stat3-driven transcription of Bcl-2 members leading to growth suppression. Indeed, STAT3 protein is either over expressed or constitutively-activated in many cancers while GRIM-19 expression is lost in many cancers (Table 1).

Effects of GRIM-19 on cell motility

A transcription-independent function for STAT3 in cell migration was also described. STAT3 interacted with Stathmin (STMN1), a microtubule disassembly factor, and promoted stability of microtubules [50]. Microtubules are polymers made from equimolar α and β tubulin monomers. Microtubules are rapidly dissociating and re-associating structures involved in multiple cellular functions including cell division and motility. Numerous post-translational modifications that occur on microtubules control their half-life and function (see [51] for a review). Apart from these modifications, the c-terminal amino acid (tyrosine) of α -tubulin is subject to periodic enzymatic removal and addition on polymerized microtubules. Removal of this tyrosine exposes the preceding glutamate residue, referred to as Glu-tubulin, and this is believed to stabilize such microtubules *i.e.*, a longer half-life. Removal of this glutamate residue renders the process irreversible and is known to occur in neurons [52]. Tubulin-containing microtentacles were reported to be essential for cancer cell metastases [53]. At present, whether GRIM-19 affects this process in epithelial-derived cancers is not known. However, in v-Src-transformed fibroblasts there was a slight decrease in the intensity of Glu-tubulin *i.e.*, microtubules are relatively more dynamic. Upon, GRIM-19 expression the intensity of Glu-tubulin was similar to the non-transformed parental cells [54]. Additionally, a significant reduction in the size of the nucleus in v-Src-transformed fibroblasts occurred, which could be attributed to cytoskeletal changes. Src activity in cells is kept silent by c-terminal Src kinase (CSK), a tyrosine kinase that phosphorylates Src at its c-terminal Y^{530/535} residue [55, 56]. In the steady state, tyrosyl phosphorylation of this residue inactivates Src tyrosine kinase. Unlike the parental Src, v-Src lacks the c-terminal region, where this critical regulatory tyrosine is present. Since v-Src lacks this site, Csk is unlikely to mediate the inhibitory effects of GRIM-19 on v-Src. In contrast, a novel Src inhibitory mechanism is exerted by a Csk-binding protein, known as phosphoprotein associated with glycosphingolipid-enriched microdomains 1 (Pag1). Pag1/Csk, complex in lipid rafts, promotes Src inhibition by phosphorylating the Y^{530/535} residue [57]. Interestingly, a recent study has shown that Pag1 alone blocks Src activity, independently of Csk [58, 59], by binding to the SH2 domain of active Src and retaining it in lipid rafts to blunt Src-driven oncogenesis. Indeed, *Pag1*^{-/-} mouse embryonic fibroblasts (MEFs) were readily transformed by v-Src, which was reversed by restoring Pag1 expression. Importantly, *CBP/PAG1* mRNA is down regulated in tumor cells expressing active Src [58]. Consistent with such observations, we noticed a strong down regulation of *Pag1* mRNA in v-Src-transformed fibroblasts, which was prevented by GRIM-19 [60]. Thus, GRIM-19 appears to indirectly inhibit v-Src activity through PAG1.

Down regulation of Inhibitor of Apoptosis Proteins (IAPs)

IAPs inhibit Caspases, which drive apoptosis. IAPs were first identified in baculoviruses and later in insects and mammals. The presence of one or more baculoviral IAP-like repeat(s) (BIRC) is a characteristic of all IAPs. In mammalian cells, IAPs prevent the activation of Caspases by blocking their active sites. Apart from this classical function, now there is ample evidence to believe that IAPs are involved in other cellular processes such as innate immunity and chromosomal segregation (see [61] for a review). The X-linked IAP (XIAP; BIRC4) blocks Caspases 3, 7 and 9. XIAP and other IAPs are in turn targeted to destruction by IAP inhibitors like second mitochondrial activator of caspases (SMAC, also known as

DIABLO) and high temperature requirement protein A2 (HTRA2) [62]. HTRA2, a serine protease located in the mitochondrial IMS, cleaves XIAP to relieve the inhibition on Caspase-9 (Fig. 2). By a physical association, GRIM-19 augmented HTRA2-driven XIAP cleavage [35]. The PDZ domain of HTRA2 and the c-terminal region of GRIM-19 were essential for such interactions. IFN/RA stimulation strongly increased HTRA2/GRIM-19 interaction, suggestive of some post-translational modification. Indeed, a dynamic phospho-T¹¹³ modification on GRIM-19 was reported using mass spectrometry [63]. The functional consequence(s) of this modification, or lacking, is unclear at present. We have shown earlier that R^{115P}, found in a certain Hürthle cell thyroid tumor was significantly incapable to blocking STAT3-driven cell growth and gene expression [64]. Curiously, this site is separated by one amino acid from T¹¹³. Whether R^{115P} affects modifications at T¹¹³ of GRIM-19; and has any bearing on Htra2 or STAT3 interactions needs to be determined. HTRA2 is upregulated in response to heat shock and/or Tunicamycin treatment and cancer cell lines do not lose expression of HTRA2 protein [65]. Additionally, HTRA2 is also involved in mitochondrial protein quality control in the nervous system as mice lacking Htra2 protein, in neurons, showed Parkinson's-like disease [66].

Another IAP member that is over expressed in cancer cells is Survivin (HGCN symbol: *BIRC5*). Survivin protein is expressed during embryonic development, and undetectable in terminally-differentiated resting cells, peaks at G₂-M phase of the cell cycle (see [67] for a review). Survivin localizes to the spindle assembly due to its interaction with microtubules. Mouse embryos lacking *Birc5* gene die prior to the implantation stage [68] similar to embryos that lacked intact *Grim-19* alleles [69]. Since, cancer cells undergo repeated cell division, Survivin is necessary for cancer cells to complete mitosis. In gynecological tumors, destruction of p53 leading to higher levels of *BIRC5* mRNA and replicative advantage was suggested [70, 71]. In primary cervical tumors, we have shown Survivin levels inversely correlated with GRIM-19 levels [72]. There was a strong correlation between STAT3 and Survivin protein levels in primary cervical tumors and xenografted cervical cancer cells expressing GRIM-19 had a lower *BIRC5* expression. Mitochondrial Survivin protein was recently suggested to drive oxidative phosphorylation [73] through RC-2 i.e., succinate dehydrogenase (SDH), similar to an earlier report of STAT3 driving such a process [74]. Interestingly, acetylated Survivin interacted with STAT3 in the nucleus to inhibit transcription of a few SIE-motif containing genes viz., *BCL-XL* and *MCL1* while it did not prevent transcription from others viz., *MYC*, *VEGF*, *CCND1* and *MMP9* [75]. Whether similar mechanisms operate in other cancers is not known at present. Given that *BIRC5* gene encodes 4 known protein isoforms; relative abundance of such isoforms and post-translational modifications on them will greatly influence cellular properties and responses.

Cell cycle control—CDKN2A (p16^{INK4A}), a cyclin-dependent kinase (CDK) inhibitor, is one of the most frequently mutated tumor suppressor genes [76]. This gene produces two alternatively spliced mRNAs that code for two completely different proteins viz., p14/19^{ARF} and p16^{INK4A}. The former interacts with MDM2 to relieve inhibition on p53 while the latter suppress the activity of CDKs 2, 4 and 6. Recently, we have shown an interaction between p16^{INK4A} and GRIM-19 using a proteomic approach [37]. When co-expressed, GRIM-19/p16^{INK4A} synergistically suppressed cell cycle progression by inhibiting E2F1-

driven gene expression. GRIM-19/p16^{INK4A} complex tightly associated with CDK4 and prevented the binding of cyclin D1 (the activator) with CDK4 (Fig. 1). The p16^{INK4A} protein contains four Ankyrin-like repeats (ARs) domains necessary for its interactions with other cellular proteins, foremost of which are the CDKs. We demonstrated that the N-terminal region of GRIM-19 interacted with the 4th AR of murine p16^{INK4A} [37]. The *CDKN2A* gene suffers multiple point mutations [77] some of which incapacitate its CDK-inhibitory function. The 1st and 2nd ARs of p16^{INK4A}, required for CDK inhibition, are frequently mutated in several human cancers. However, the significance of mutations in the 4th AR, a domain not required for CDK4 inhibition, was unclear. Our studies showed that mutations in the 4th AR, found in certain human adenocarcinoma and squamous cell carcinoma (SCC) of lung, prevented the interactions between p16^{INK4A} and GRIM-19 [37]. This seems to lead to an inefficient CDK4 inhibition, which permits cell cycle progression.

OLFM4 (GW112), an extra-cellular matrix (ECM) glycoprotein from the Olfactomedin family, is abundantly expressed in inflamed colonic mucosa, gastric, pancreatic and colorectal cancers [34] and promoted growth of sub-cutaneously implanted syngeneic mouse prostate cancer cells. A later study suggested that this protein promoted S-phase transition in pancreatic cancer cells [78]. Even though the region(s) involved in binding was not mapped, interaction occurred primarily in the mitochondria when both proteins were over expressed (Fig. 1). *Olfm4* gene-deleted mice develop normally and do not show a discernible phenotype [79]. In a gastric cancer cell line, expression of GRIM-19 suppressed GW112 levels in addition to VEGF, MMPs 2 and 9. Similar to GRIM-19 acting as an inhibitor of STAT3, this study suggested GRIM-19 could also inhibit NF- κ B activation using an *in vitro* assay [80]. However, other reports have argued OLFM4 to be a tumor suppressor in murine melanoma [81] and human prostate cancer [82]. Hence, the role of OLFM4 in other cancers needs to be critically evaluated. Later studies have demonstrated OLFM4 to regulate innate immune responses. Reduced colonization by *Helicobacter pylori* in mice lacking *Olfm4* gene suggested Olfm4 to be an inhibitor of mucosal immunity [79]. Subsequent studies by the same group demonstrated Olfm4 inhibited cathepsin-C and in the absence of Olfm4 protein, neutrophil-driven innate immune responses against *Staphylococcus aureus* and *Escherichia coli* [83] increased significantly but not against *Aspergillus fumigatus* [84]. In summary, it is not clear why and how an ECM protein exerts a prominent role in nuclear transcription and the physiologic relevance in such contexts.

Innate anti-bacterial responses induce GRIM-19—Pattern recognition receptors (PRRs) are crucial for detecting pathogen-associated molecular patterns (PAMPs) and activating appropriate counter measures to control the invader. In vertebrates, the Toll-like receptors (TLRs) [85], C-type lectin receptors (CLRs) [86], Nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) [87] and Retinoic acid-inducible gene (RIG)-like receptors (RLRs) [88] detect distinct molecular signatures to activate innate immune responses. The intracellular NLR NOD2 recognizes bacterial muramyl dipeptide and activates NF- κ B to boost innate immune responses [89]. Members of the TLR family and RIPK2 (a serine-threonine kinase), are believed to be important in this signaling cascade. RIPK2 is necessary for innate and acquired immune responses and the c-terminal caspase-recruitment domain (CARD) may be important for this function. Even though the

GRIM-19 residues crucial for interaction with NOD2 are not known, the N-terminal CARD15 domain of NOD2 was not required for this interaction [36]. Surprisingly, cytoplasmic GRIM-19 could protect host cells by decreasing the survival of intracellular *Salmonella typhimurium*. This study showed an interaction *in situ* using over-expressed proteins. In non-immune cells, intra-cellular *S. typhimurium* could up regulate *GRIM-19* mRNA levels whereas non-pathogenic *E. coli* did not. *In vitro* cultured human macrophage-like cells up regulated GRIM-19 protein level when challenged by purified cell wall fraction of *Mycobacterium bovis* BCG [90]. In peripheral blood-derived monocytes, however, only a live bacterium (*Porphyromonas gingivalis*) not its LPS or fimbrial proteins, could up regulate GRIM-19 protein levels [91]. Aged *Grim-19^{+/-}* male mice were prone to spontaneous obstructed urinary tract infection, mostly by *Staphylococcus saprophyticus* [92] compared to wild-type mice. Such an innate immune response is also conserved in insects as a differential response and enrichment of *GRIM-19* expressed sequence tags were observed in hemocytes from mosquitoes (*Armigeres subalbatus* and *Aedes aegypti*) when challenged with *E. coli* and *Micrococcus luteus* [93]. Thus, GRIM-19 may serve as an anti-bacterial protein by stimulating cytokine synthesis through the “Inflammasome”. This ability of intracellular bacteria may have contributed to their anti-tumor effects as we noted that naïve *S. typhimurium* reduced tumor growth compared to mock inoculations [94]. We have also shown that recombinant *S. typhimurium* carrying plasmids encoding *STAT3*-specific shRNAs strongly reduced tumor burden and metastases [94]. In these experiments, upregulation of GRIM-19 along with blunting of *STAT3* appear to occur simultaneously. A recent study reported delivery of GRIM-19 to tumors, using attenuated *S. typhimurium*, inhibited growth of xenografted tumors better than individual agents in murine hosts [95]. It is still not clear as to how GRIM-19 is induced by PRR-initiated signaling in a very short time given that it takes a much longer time for GRIM-19 to be induced by RA/IFN- β [2] or a high dose of IFN- β alone in non-immune cells (Fig. 3).

Inhibition of GRIM-19 by viruses

Viruses employ several strategies to attenuate, subvert or evade host innate immune responses. Human herpesvirus-8 encoded gene products like vIL-6, vBcl2 and vIRFs are classic examples of this type [96]. Viral IRF1 (vIRF1), encoded by the human herpesvirus 8 (HHV-8), inhibits IFN gene expression by preventing with the interaction of CBP/p300 with co-activator IRF3. Since GRIM-19 is an IFN-inducible gene product (Fig. 3), blocking IFN synthesis gives time for the virus to take control of the cellular machinery. Additionally, vIRF1 also binds to GRIM-19 and prevents its ability to induce apoptosis [97]. A closely related human herpesvirus 6B (HHV-6B) employs a different protein, U95, to block GRIM-19-induced apoptosis [98]. The E6 protein of high-risk human papillomaviruses (HPV) binds to GRIM-19, but E6 protein from the low-risk strains does not [97]. More importantly, in primary cervical cancers, loss of GRIM-19 associated with a loss of p53 protein [99]. The HPV E6 oncoprotein alters the specificity of a host ubiquitin ligase E6AP (HGCN symbol: *UBE3A*) to accept p53 as a substrate [100] for ubiquitylation that is degraded by the proteasome. In high-risk HPV-positive cancer cell lines, GRIM-19 prevented the association of HPV-18 E6 with E6AP to form a complex and restored p53 protein levels [99] to induce apoptosis (Fig. 2). Interestingly GRIM-19 could induce the auto-ubiquitylation of E6AP to be degraded by the proteasome [99] (Fig. 2). Additionally,

E6-E6AP complex can induce transcription of the telomerase gene (HGCN symbol: *TERT*) to maintain chromosomal end stability [101] and hence replicative immortality. In our recent report, we have shown that TERT levels inversely correlated with GRIM-19 levels in primary cervical SCCs [102]. Human cytomegalovirus (HCMV) employs a non-coding RNA ($\beta 2.7$) to prevent release of GRIM-19 from mitochondria [103]. Viral mutants lacking $\beta 2.7$ were unable to prevent apoptosis. Even though the entry of $\beta 2.7$ into the mitochondria was reported to be independent of viral protein factors, the mechanism still remains elusive as to how large nucleic acids can traverse mitochondrial membranes. Vaccinia virus was reported to downregulate *GRIM-19* mRNA as early as 2h post infection and such low levels were maintained for as long as 16h [104]. The factors involved in GRIM-19 down regulation are not known. In summary, a majority of the DNA viruses block GRIM-19 for promoting the survival of virus-infected or virus-transformed cells (Fig. 3).

The mitochondrial function of GRIM-19

The mammalian RC-1, comprised of ~46 distinct proteins [105], is the largest of RCs. However, only 14 proteins in this complex are critical for catalysis *i.e.*, electron transport and proton pumping. Seven each of these proteins originate from the nuclear and mitochondrial genomes. Bacteria by far have the simplest RC-1, that is embedded in the plasma membrane and pumps protons into the periplasmic space to drive oxidative phosphorylation *i.e.*, ATP synthesis. However, bacterial RC-1, the prototype, contains only 14 proteins with catalytic activity; same as in simpler eukaryotes to highly complex mammalian cells. This raises a question - why does the mammalian RC-1 contain additional proteins that have no catalytic activity? A probable answer is that mammalian RC-1 catalytic core (14 proteins) is smaller in size compared to bacterial RC-1 and the additional 32-plus 'accessory proteins', may be required to stabilize this complex. Experimental approaches to address this question have not yielded convincing answers yet, as removal of even a single 'accessory protein' drastically interfered with the maturation of mammalian RC-1 and triggered cell death [69, 106]. Additionally, low levels of mature RC-1 due to mutational inactivation of assembly factors manifested as cell death [107, 108] or tissue-restricted vulnerability [109] or kinetic alterations [110]. A bewildering situation is seen in budding yeast (*Saccharomyces cerevisiae*) that lacks 'true' RC-1 and GRIM-19 orthologue; yet it does not exhibit defects in oxidative phosphorylation or cell viability. This observation suggests that cells can compensate mitochondrial NAD^+/NADH redox reaction by other mechanisms. A recent study showed transgenic expression of Ndi1, the alternative NADH dehydrogenase in *S. cerevisiae*, could rescue the embryonic lethal phenotypes of *Caenorhabditis elegans* depleted of RC-1 proteins and such worms were rotenone insensitive like the yeast enzyme [111]. In an earlier study, Ndi1 was found to protect cardiomyocytes from ischemia-reperfusion (IR) injury-induced damage [112]. During IR, mitochondrial dysfunction, especially RC-1, generates ROS that is toxic to cells. We reported that GRIM-19 was upregulated during focal ischemia-induced damage in rat brain [113] [113][2] and neurons were more sensitive compared to glial cells. Whether such mechanisms mentioned above would suppress cancer cell growth needs to be critically examined.

Structural studies have shown that RC-1 conforms into an 'L' shaped structure [105]. The hydrophobic arm of RC-1, composed of the proton-translocating subunits (7 proteins; ND1,

2, 3, 4, 4L, 5 and 6) encoded by the mitochondrial genome in eukaryotes, is embedded in the inner mitochondrial membrane (IMM). The hydrophilic arm (7 proteins; NDUF51, 2, 3, 7, 8 and NDUFV1, 2) that projects into the mitochondrial matrix contains the redox centers necessary for electron transport. A recent single-particle electron cryo-microscopic analysis mapped a trans-membrane helix of GRIM-19 wrapping ND1 while NDUF8 formed an L-shaped clip over GRIM-19 [114]. Using dissociative methods, however, GRIM-19 was demonstrated to be a part of sub complexes 1 α or 1 β , while ND1 was in sub complex 1 λ [115]. Further studies are needed to ascertain the spatial relationship among the RC-1 subunits. Mammalian RC-1 was shown to be assembled in a modular fashion in immortalized cell lines using an epitope-tagged protein [115]. If the same mechanism operates in normal cells needs to be evaluated. Functional consequences of an 'accessory protein' depletion manifests as drastic reduction in mature RC-1 levels concomitant with accumulation of submodules or intermediates. A very low level of mature RC-1 is insufficient to maintain effective transmembrane potential and, as a result, triggers cell death [116]. Consistent with this notion, direct deletion of mouse *Grim-19* gene revealed an embryonic lethal phenotype [69] as no viable embryos were recovered beyond the implantation stage *i.e.*, blastocyst. Mitochondria are important for calcium homeostasis, especially in excitable tissues like neurons and muscles. A morpholino-based GRIM-19 depletion disrupted calcium signaling in *Xenopus laevis* embryos that led to defective heart development [117]. We have shown that conditional deletion of *Grim-19* in adult mouse skin does not cause significant lethality. However, these mice are highly prone to chemically-induced tumor development [118]. Even, a mono-allelic loss was sufficient for tumor development. A significant decline of mitochondrial electron transport machinery viz., RC-2, RC-4 (cytochrome c oxidase) and RC-5 (ATP synthase) and a modest increase in RC-3 (cytochrome c reductase) levels occurred in the absence of GRIM-19 [118]. These are consistent with a strong decline in oxygen consumption in these cells. A recent study reported that GRIM-19 point mutation R⁵⁷H in two sisters of family, was associated with the development of hypotonia, dyskinesia and sensorial deficiencies, including a severe optic neuropathy [119]. These patients have been shown to have RC-1 instability and defects in mitochondrial electron transport. Thus, GRIM-19 plays a prominent role in mitochondrial oxidative phosphorylation.

Functional consequences of GRIM-19 defects: their relevance to cancer

Based on several independent studies in many types of tumors, advanced tumors express extremely low level of GRIM-19 protein compared to benign tumors (Table 1). GRIM-19-deficient tumors express high STAT3 levels and STAT3-responsive gene products compared to adjacent normal tissue. Notably, elevated mRNA expression level of anti-apoptotic Bcl2 family members were noticed in these samples [46, 72, 120, 121]. GRIM-19 is required for the inhibition of STAT3-induced oncogenic gene expression is a common observation from these studies (Table 1). The mitotic cyclins and CDKs were also elevated, due to loss of CDK inhibitors. The negative regulators of tumor suppressors were elevated leading to negligible growth-suppressive protein levels. Mutational inactivation (genetic deletions or point mutations) or epigenetic suppression of tumor suppressor genes is commonly observed in human cancers. Compared to the frequency of mutant *TP53* and *CDKN2A*, mutation(s) in

genes encoding mitochondrial ETC proteins are rare. Mutant *GRIM-19* gene is represented at a lesser frequency in COSMIC database at the time of writing this article (Table 1). It should be noted that *GRIM-19* genetic alterations may not have been documented, owing to a lack of deeper analyses of human tumors for *GRIM-19* at this time. In addition, relative to *TP53* and *CDKN2A*, *GRIM-19* is new player on the arena. It is expected that future studies will identify more genetic alterations and provide vignettes of GRIM-19 as a major tumor suppressor. We have studied the functional consequences of 5 tumor-derived mutations *in vitro* and *in vivo*. Two point mutations (K⁵N and R¹¹⁵P) were reported in thyroid tumors by genomic DNA sequencing [122]. We have analyzed numerous kidney, head and neck SCC (HNSCC), cervical and prostate tumors using patient-matched normal controls at the RNA, DNA and protein levels for GRIM-19, STAT3 and other growth-regulatory genes. We identified three *GRIM-19* point mutations (L⁷¹P, L⁹¹P and A⁹⁵T) in a HNSCC cohort [46] who had long history of tobacco usage (Table 1). In addition, human cancer cell lines harbor a unique *GRIM-19* mutation (Table 1). The ENSEMBL database lists D⁹, L⁷¹ and L⁹¹ residues of GRIM-19 to be mutated, using low-resolution techniques, in tumor samples but the source was not described. We had reported the N-terminal QDMP motif, residues 8–11, of GRIM-19 to be important for its growth-regulatory effects [123], especially the D⁹ residue. We have observed this residue to be mutated in a HNSCC cell line (HN-12) which we recently obtained. HeLa cells harbor K⁷E mutation in a heterozygous state while HNSCC cell lines (HSC2 and HSC3) lack c-terminal two amino acids due to a mutation in codon 143 (TAC → TAG; Y → Stop) leading to premature translational termination. The point mutations described in COSMIC database are of a heterozygous nature obtained by high-resolution DNA sequencing. Two point mutations, R⁵⁷C and R¹¹⁵C, mentioned in the COSMIC database appear to be very interesting for a protein that lacks cysteine residues. Such a mutation could potentially subject GRIM-19 protein for thiol-redox regulation in an intra-molecular and/or inter-molecular fashion.

Certain cancers e.g., renal cell carcinomas (RCCs), pheochromocytomas and paragangliomas show a unique mutational spectrum. Point mutations in TCA cycle enzymes and/or mitochondrial OXPHOS coding genes are common feature. A mutation in fumarate hydratase (FH) is characteristic of papillary-type RCC [124] while subunit C [125] or D [126] of SDH is mutated in pheochromocytomas and paragangliomas. These genes are rarely mutated in other cancers. Our report of GRIM-19 loss-of-expression in RCC using mass spectrometry [120] and other quantitative methods added a new dimension to mitochondrial dysfunction in this cancer. By analyzing tumor and patient-matched adjacent normal tissue, clear cell RCC samples had severe loss of GRIM-19 expression compared to other types of RCCs and/or urogenital tumors. Since most of these samples came from sporadic cases, tumorigenic events appear to be independent of von Hippel-Lindau (VHL) protein. VHL is an ubiquitin ligase that marks the labile component of hypoxia-inducible factor (HIF) for degradation during normoxia [127]. HIFs are transcription factors activated by low oxygen concentration (reviewed in [128]). Functional HIF-1 is a heterodimer consisting of HIF-1 α and HIF-1 β (HGNC symbol: *ARNT*) proteins while HIF-2 is composed of HIF-2 α and HIF-2 β (HUGO symbol: *ARNT2*). The alpha subunit of HIF-1 and HIF-2 are rapidly and constantly degraded when sufficient oxygen is available, while the beta subunits are not. When oxygen is present, alpha subunits are hydroxylated by specific

prolyl-hydroxylases (PHDs) at critical proline residues leading to ubiquitination by VHL and destruction by the proteasome. Low oxygen levels inactivate the PHDs leading to the stabilization of HIF-1 and HIF-2. The gene products regulated by HIF-1 or HIF-2 are important for cells to survive periods of oxygen deprivation (see [128] for additional details). In addition to some of the glycolytic enzymes upregulated by HIF-1 [129, 130], modifying RC-4 (cytochrome c oxidase; also known as COX) activity appears to be a prominent response to hypoxia [131]. During hypoxia, HIF-1 upregulates transcription of two genes viz., Lonp1 and Cox4i2. Lonp1 is a protease that degrades Cox4i1 in the IMS to be subsequently replaced by Cox4i2. Interestingly oxidized aconitase, an enzyme in the mitochondrial matrix, is also degraded by Lonp1 [132]. Whether such changes improve mitochondrial ETC efficiency or prevent ROS generation or decrease cellular respiration rate during hypoxia has not been critically examined.

GRIM-19 mutations: influence on cancer cell characteristics

We have studied how tumor-derived mutant GRIM-19 proteins affect cellular functions when ectopically expressed in cell lines that have low levels of endogenous GRIM-19 protein. When a mutant GRIM-19 is expressed, cells grow rapidly compared to cells expressing wild-type GRIM-19. We have shown by *in vitro* and *in vivo* experiments that mutant GRIM-19-expressing cells have higher levels of cell-cycle associated gene products like cyclins D1, B1, CDKs, DHFR, TK1 etc [37, 46, 123]. Importantly, GRIM-19 point mutants do not interact as strongly as wild-type GRIM-19 does with STAT3. Since STAT3 cannot be blocked efficiently by mutant GRIM-19, cell growth was not restrained by them. Even though these point mutations are well separated in the primary sequence, whether they appear to contribute to the STAT3-interacting surface or contact points.

Cell survival

Many oncogenic transcription factors, like HIF-1 α , NF- κ B, E2F1, DP1 *etc*, are also upregulated during tumor progression and are required for transcriptional induction of genes involved in cell division. Aberrantly high STAT3 activity in cancer cells can be due to EGFR gene amplification or loss of feedback inhibitors like SOCS3, PIAS3 and GRIM-19. The Src protein, a non-receptor tyrosine kinase (NTK), localizes to plasma membrane and associates with different proteins. Src activity is controlled by phosphorylation of two residues in its kinase domain i.e., the c-terminal region. Phosphorylation of the Tyr⁴¹⁶ (419 in humans and 424 in rodents) renders the kinase active while phosphorylation of the Tyr⁵²⁷ (530 in humans and 535 in rodents) renders the kinase inactive. As v-Src lacks the last ~11 amino acids, it can function as a strong (Tyr⁴¹⁶ is phosphorylated) kinase and escapes negative regulation by CSK. Although *Src* is not mutated at high frequency in human tumors, Src activities increase as tumors progress. Solid tumors cannot grow beyond a particular size due to limited nutrient availability. Due to the low oxygen saturation in tumors (>50mm³), HIF-1 α gets stabilized and tumors start secreting high levels of VEGF to form new blood vessels, which helps them to grow and metastasize [133]. Upon nutrient availability, nucleic acid biosynthetic enzymes (DHFR, TK1 etc) are upregulated leading to cell proliferation. At this time, matrix metalloproteases (MMPs) are also secreted to reshape the tumor microenvironment [134]. STAT3 and HIF-1 α are known to drive up transcription of certain

MMPs and induce blood vessel formation (see [135] for a review). We have shown a significant suppression of new blood vessels in xenografted tumors expressing GRIM-19, relative to the control tumors without GRIM-19 [72]. GRIM-19 also suppressed MMP expression in these tumors. One of the substrate for MMP-7 is the FAS ligand (FASL) [136]. Membrane-bound FASL induces apoptosis while the soluble FASL is known to be mitogenic to cancer cells (see [137] for a review). In addition, MMP-7 activates MMP-2 and MMP-9 to promote matrix degradation [138]. Thus a decrease in MMP-7 could have directly halted mitogenic signaling and invasion.

Cell proliferation

As mentioned earlier, cyclin-bound CDKs phosphorylate retinoblastoma (RB) and RB-like proteins [139] to remove the inhibition on E2F1 transcription factor. Removal of RB releases E2F1 protein and its association with DP1 to form transcriptional complexes at specific gene promoters of genes involved in G₁-S phase transition [140]. This check point is also regulated by inhibitors of CDKs, comprising the CDKN1 (Cip and Kip) and CDKN2 (Ink4) family members (see [141] for a review). Many cancers lose expression of these tumor suppressors [142]. Loss of interaction(s), with cyclins and/or CDKs, due to point mutations in the N-terminal and middle region of the protein was easier to observe. All CDKN2 members have Ankyrin-like repeats (ARs) in their tertiary structure. ARs are all α -helical structures composed of ~35-amino acid-long sequences that are arranged in anti-parallel fashion. Such repeat organization is believed to mediate protein-protein interactions. The significance of point mutations observed in the 4th AR and c-terminal region of p16^{INK4A} was unclear. We showed that this region is important for interaction with other tumor suppressors, like GRIM-19. The impact of point mutations observed in glioblastomas and NSCLC (see [77] for complete list), the c-terminal region of human p16^{INK4A} was analyzed in terms of GRIM-19 interaction. Tumor-derived p16^{INK4A} mutants G¹³⁹D and A¹⁴⁷G failed to interact with GRIM-19 while mutants S¹⁴⁰C and H¹⁴²R significantly lost their ability to bind to GRIM-19, compared to wild-type p16^{INK4A} protein [37]. Hence, cell cycle is not tightly controlled as the wild-type protein(s).

Cell motility

Cytoskeletal reorganization is necessary for cancer cell motility. Plasma membrane resident FAK and Src are important players in cancer cell adhesion and detachment [143]. Highly metastatic cells have weak contact with the substratum *i.e.*, ECM, and detach readily [144]. This could also be due to a rapid turnover of focal contacts, which otherwise would keep them adherent. FAK activity is crucial for focal adhesion turnover while Src is dispensable for this process [145]. In v-Src-transformed cells, tyrosyl-phosphorylated FAK, paxillin and γ -catenin levels were higher than control cells that resulted in increased motility, and this occurred independently of STAT3. A dynamic actin network is controlled by many primary regulators in a spatio-temporal pattern (see [146] for a review). GRIM-19 controlled the actin regulators to suppress cytoskeletal remodeling. GRIM-19 controls the level of Cortactin, a secondary actin regulator. Src-mediated phosphorylation of Cortactin reduces its affinity for actin and Cortactin's ability to stabilize Y-branched actin networks. This triggers reorganization of cortical actin networks (Fig. 4). Upon expression of wild-type GRIM-19 in v-Src-transformed cells, actin stress fibers were regenerated with a suppression of

podosomes / filopodia / lamellipodia suggesting reversion to a non-transformed state [54]. This reversion was also accompanied by a reduction in phospho-Y⁴¹⁶ v-Src levels without affecting total v-Src levels. Such suppression was not due to a physical interaction between v-Src and GRIM-19. The N-terminus of GRIM-19 was essential for this inhibitory process. As mentioned earlier, v-Src is not subject to negative regulation by CSK and the mechanism for reduced phospho-Y⁴¹⁶ v-Src levels was enigmatic. Since, the Y⁴¹⁶ residue is in the kinase domain, we first checked for negative regulators of tyrosine kinase signaling. Among the negative regulators of tyrosine kinase signaling, CBL family members were the first suspects. CBL proteins are ubiquitin ligases involved in cell signaling. Two CBL members, c-Cbl and Cbl-b, are expressed by all cell types while the third member Cbl-c is primarily expressed by epithelial cells. We initially hypothesized Cbl-mediated v-Src ubiquitination as a possible mechanism for the reduction in phospho-Y⁴¹⁶ v-Src levels upon GRIM-19 expression. Instead a CSK-binding protein present in lipid rafts known as Pag1 suppressed active levels of v-Src (Fig. 4). Indeed, *Pag1*^{-/-}MEFs showed an increased susceptibility to v-Src-induced transformation. It is likely that Pag1 physically interacts with Src in lipid rafts or microdomains before being targeted to specific plasma membrane foci. Therefore, a fraction of v-Src protein was sequestered from gaining the crucial phosphorylation on Y⁴¹⁶ that makes the tyrosine kinase very active. Tumor- derived GRIM-19 mutants could not upregulate Pag1 levels like wild-type GRIM-19, leading to the persistence of v-Src activity and unabated cytoskeletal remodeling [60].

Cell invasion

Epithelial-Mesenchymal Transition (EMT) is characteristic of tumor cells undergoing local invasion to lymph nodes or distant metastases. When epithelial cells begin to express more of mesenchymal genes concomitant with reduced expression of epithelial genes, the process of EMT ensues (see [147] for a review). This adaptation contributes two important advantages to transformed epithelial cells – 1) it makes them resistant to Anoikis, rapid death induced due to loss of interaction with the ECM [148], and 2) it allows them to migrate short distances from the site of origin [149]. With time, the migrated tumor cells acquire additional characteristics that transform them into aggressive state of growth and migration. Presence of Vimentin (mesodermal-type intermediate filament) and reduction in epithelial-type cadherin (HGCN symbol: *CDH1*; commonly known as E-Cad) are classic markers to score for EMT *in situ*. We have observed EMT-like changes in S3C-expressing HaCat cells (Nallar and Kalvakolanu, unpublished); the cells acquired a spindle shape and were less adherent. Consistent with this, upon expression of GRIM-19, in S3C-expressing cancer cell lines, metastatic spread and tumor burden were reduced in nude mice. Tumor-derived GRIM-19 mutants were less effective at controlling metastases compared to wild-type GRIM-19 [46]. Thus, GRIM-19 exerts anti-metastatic effects.

Loss of GRIM-19 increases susceptibility to tumor development

As a direct *Grim-19* gene deletion is incompatible with mouse development, we have generated a genetically modified mouse in which *Grim-19* gene could be conditionally deleted [118]. *Grim-19* gene was deleted in the mouse skin using a tamoxifen-inducible Cre recombinase. No spontaneous tumors developed in gene-deleted mice over a period of 18-

plus months. However, these mice were prone to DMBA/TPA-induced skin carcinogenesis. Earlier tumor appearance, increased number and size of tumors were commonly observed in *Grim-19*-deficient mice compared to mice with two intact copies of *Grim-19* allele. Importantly, mice with a mono-allelic loss of *Grim-19* showed increased susceptibility to chemical carcinogenesis. Major findings of our study were presence of high nuclear cyclin D1 protein [150] and up regulated levels of membrane-associated β -catenin proteins [151] in *Grim-19*-deficient tumors [118]. In addition to phosphorylated Stat3, Ezh2 protein was up regulated in *Grim-19*-deficient tumors. Steady-state transcript levels of *Sox2*, cyclins *B1* and *D1*, *Cdk1*, *Dhfr* and *Bcl2-11* levels were also up regulated. Glucose utilization was uncoupled from TCA cycle and more lactate was excreted by *Grim-19*-deficient cells. As mentioned previously, oxygen consumption decreased in *Grim-19*-deficient cells. Mitochondrial RC-3 levels increased in *Grim-19*-deficient cells while the levels of all other complexes (RC-1, -2, -4 and -5) decreased [118]. This observation is in agreement with similar defects observed in cancer cell lines [60]. To compensate for such changes, transcript levels of glycolytic enzymes Aldolase-A (*Aldoa*) and Pyruvate kinase M2 isoform (*Pkm2*) increased in *Grim-19*-deficient tumors. Interestingly, transcript levels coding for protein regulators of glycolytic enzymes viz., Pyruvate dehydrogenase kinase (*Pdk2*) and Glucokinase regulator (*Gckr*) are also increased in *Grim-19*-deficient tumors. Treatment of *Grim-19*-deficient tumor-bearing mice with a Stat3 inhibitor blocked tumor growth indicating a prominent role for Stat3 in tumor development [118]. Also, the most common gene mutation documented in such skin tumors i.e., *Ras* members, was not altered following the loss of *Grim-19*.

As the tumor grows, cancer cells also face nutrient competition. Upon reaching a certain critical size at the primary site, the cells near the center of the mass experience low oxygen levels and reduced nutrient delivery and waste removal. Such cells either undergo a necrotic cell death or synthesize cell survival factors or enter into a state of quiescence (reviewed in [152]).

The first scenario of necrotic cell death induces tumor inflammation. Cell-derived proteins and nucleic acids act as inflammatory stimuli for the resident macrophages, promote the production of several pro-inflammatory cytokines like IL-1, IL-6, TNF- α , CCL2, CXCL1, CXCL8. This further leads to recruitment of other immune cells from systemic circulation and an array of cytokines are secreted further [153]. This process has the potential to remodel the tumor microenvironment. Inflammatory signals alter the tissue milieu by recruiting cells of immune system. Acute inflammatory reactions to bacterial presence in tumor microenvironment resulted in tumor lysis (see [154] for a review). GRIM-19 was upregulated during anti-bacterial responses and tumor lysis could be enhanced when such bacteria delivered GRIM-19 expression plasmid to the tumor [155]. However, GRIM-19 was not upregulated in chronic inflammatory conditions like arthritis [156] and IBD [36]. In such chronic conditions, a recent study showed a higher GRIM-19 level to be beneficial. In collagen-induced mouse arthritis model, GRIM-19 suppressed disease progression by decreasing Th-17 and increasing T-reg cell population [156] and inflammatory cytokines (TNF- α , IL-1 β , IL-6 *etc*) levels were attenuated in such joints. In this study, GRIM-19 transgenic mice (C57BL/6 background) and GRIM-19 over expressed (DBA1/J background) by intra-venous or intra-muscular administrations of an expression vector were used.

Earlier publications demonstrated the presence of mitochondrial STAT3 in Ras-transformed cells [157], pro-B cells [158], mouse myocytes and hepatocytes [74]. In these studies the authors suggested STAT3 interacted with RC components to sustain or prevent defective oxidative phosphorylation. With the knowledge that these experiments were conducted in different cell types, these reports appear to contradict the function of STAT3-GRIM-19 interaction in mitochondria although all needed Ser⁷²⁷ to be phosphorylated. However, we have not observed mitochondrial STAT3 in our experiments to date (Kalakonda and Kalvakolanu, unpublished). More importantly, GRIM-19 is present in RC-1 at the expected stoichiometric level while the STAT3 protein was far less represented in heart mitochondrial preparations [159]. It seems STAT3 can regulate RC-1, -2 and -5 by a physical interaction. Thus, the relationship between mitochondrial STAT3 (only in some cell types) and GRIM-19 is not clear at this time. Importantly, it is not clear whether STAT3 in the mitochondria promotes tumor inflammation and whether this inactivates and/or polarizes immune cells in the tumor microenvironment.

In the second scenario of survival, due to lower oxygen availability, HIF-1 α gets stabilized and secretion of vasculogenic growth factors (VEGF, PDGF, etc.) leads to angiogenic and/or neovascular programming [160]. Formation of new blood vessels helps deliver nutrients and drain waste products but also brings in oxygen that would destabilize HIF-1 α . Hence, this process would work like an on-off switch depending on the status of negative regulators of HIF proteins. This process does remodel the tumor microenvironment extensively [161]. Clinically, these types of tumors exhibit aggressive behavior due to cyclical hypoxia. We have shown the inhibitory effect of GRIM-19 on STAT3-driven [72] and v-Src-driven [60] vasculogenesis.

In the third scenario of quiescence, or dormancy, all cellular processes are kept operational at a very minimal rate (see [162] for a review). Clinically, these type of tumors show chronic hypoxia. This would make them similar to resting stem-like cells that are resistant to standard radiotherapy or chemotherapy (see [163] for a review) due to diffusional barrier resulting in very low oxygen and drug levels, respectively. It is important to note that resistance in these cells is not due to a loss of apoptotic machinery. Asymmetric division by a few cancer cells in a tumor mass was recently described to result in cancer dormancy [164].

Another hall mark of many advanced tumors is aerobic glycolysis. Initially described by Otto Warburg, the Warburg effect describes a preferential derivation of energy by non-oxidative breakdown of glucose even when sufficient oxygen is present [165]. Later on, the Warburg hypothesis postulated that the driver of tumorigenesis was insufficient cellular respiration [166]. As our understanding of cellular metabolism has improved, an important requirement for cells to maintain a constant proliferative state is to minimize using glucose-derived carbons as an energy source *i.e.*, to prevent pyruvate from entering the TCA cycle. Although this observation was made a century ago by Otto Warburg, defective mitochondria were blamed for “Aerobic Glycolysis”. Now it is becoming clearer that this metabolic adjustment primarily serves to support nucleic acid synthesis and subsequent cell division by routing glucose through the pentose phosphate pathway (PPP) to provide ribose and sustain high nucleotide pool in cell culture conditions [167]. This pathway generates NADPH that

could be used for membrane lipid synthesis and/or restore oxidized cellular components. An interesting question to ask is whether aerobic glycolysis i.e., lactate excretion, is temporally separated from nucleotide biosynthesis? We suggest that to divert glucose-derived carbon into biomass, a minimal buildup of lactate may be required to signal cells to start DNA synthesis and/or alternate uses of glucose in hexosamine synthesis.

Loss of GRIM-19 affects glycolytic gene expression

The first step of glycolysis catalyzed by hexokinase (HK) converting glucose to glucose-6-phosphate (G6P) is irreversible. In mammals, four genes that code for HKs (*HK1*, 2, 3 and 4 (HGCN symbol: *GCK*)) are expressed in a tissue-dependent manner with distinct enzymatic and regulatory properties (see [168] for a review). In cancer cells, HK2 is the dominant isozyme [169] and is induced by a variety of stress conditions, while HK1 serves a house-keeping function. In *Grim-19* deleted primary MEFs or skin tumors, *Hk1* and *Hk2* transcript levels do not vary much compared to controls (Fig. 5). G6P can now enter into the PPP or isomerized to fructose-6-phosphate (F6P) in the second step of glycolysis catalyzed by phosphoglucose-isomerase (PGI). Additional functions have been described for this protein (see [170] for a review). The next step in glycolysis is rate limiting where F6P is converted to fructose-1, 6-bisphosphate (FBP (1, 6)) catalyzed by phosphofructo-kinase (PFK). This reaction is controlled by multiple cofactors, regulatory proteins and pH (see [171] for a review). In mammals, three genes coding for *PFKL* (liver) *PFKM* (muscle) and *PFKP* (platelet) with tissue-enriched expression patterns and differences in enzymatic and regulatory properties are known [172]. In primary MEFs upon *Grim-19* deletion, *Pfkl* transcript levels are high [118] while *Pfkm* and *Pfkp* do not vary much (Fig. 5). The PFKs need fructose-2, 6-bisphosphate (FBP (2, 6)), an allosteric activator produced by phosphofructo-kinase/fructose bisphosphatase (PFKFB), to generate FBP (1, 6) from F6P. In mammals four genes coding for *PFKFB* (1, 2, 3 and 4) are ubiquitously expressed with tissue-dependent high-low expression patterns (see [173] for a review). PFKFBs are mixed function enzymes i.e., a kinase and a phosphatase. This is believed to control the flux of F6P for catabolic or anabolic processes. In *Grim-19* deleted primary MEFs, *Pfkfb4* transcript levels are low [118] while the others are not affected (Fig. 5). Splitting FBP (1, 6) into two 3-carbon molecules viz., glyceraldehyde-3-phosphate (GAP (3)) and dihydroxyacetone phosphate (DHAP), is catalyzed by aldolase (ALDO). In mammals two genes (*ALDOA* and *ALDOC*) are ubiquitously expressed while the third is liver-specific (*ALDOB*) [174]. *ALDOA* and *ALDOC* levels are highest in muscle and nervous tissues, respectively while other tissues have their unique 'high or low' expression patterns. In cancer cells, *ALDOA* expression is high [175]. In *Grim-19*^{-/-} skin tumors, *Aldoa* mRNA levels are high [118] (Fig. 5). The next reaction catalyzed by GAPDH is primarily dependent on the availability of NAD⁺. This is indirectly controlled by triose-phosphate isomerase (TPI) as the reaction equilibrium favors DHAP over GAP (3) [176]. So when enough NAD⁺ is available, GAP (3) is converted to bis-phosphoglycerate (BPG (1, 3)) by GAPDH. Cells have two ways of regenerating NAD⁺ viz., the glycerol-phosphate and malate-aspartate shuttle. Both shuttles need mitochondria to be functional. Depending on its cellular location, GAPDH has many other functions [177]. The first ATP molecule to be produced in glycolysis is catalyzed by phospho-glycerate kinase (PGK) producing 3-phosphoglycerate (PG (3)) from BPG (1, 3).

In mammals, PGK1 is expressed by all cells while PGK2 is expressed only in the testis [178]. The PG (3) produced by PGK is isomerized to 2-phosphoglycerate (PG (2)) by phospho-glycerate mutase 2 (PGAM2) [179]. In mammals, PGAM1 expression is restricted to brain and testis while homologs PGAM4 and PGAM5 are expressed by most tissues. It is not exactly clear what PGAM4 and PGAM5 proteins do in the cell. In primary MEFs, upon *Grim-19* deletion *Gapdh*, *Pgk1* and *Pgam2* transcript levels are not affected in a significant manner (Fig. 5). The dehydration of PG (2) yields phosphoenolpyruvate (PEP), a reaction catalyzed by enolase (ENO). In mammals, three genes coding for *ENO1* [180], *ENO2* (nervous enriched) [181] and *ENO3* (muscle enriched) [182] are known. In *Grim-19* deleted primary MEFs, *Eno3* transcript levels dropped steeply (Fig. 5). The next irreversible step in glycolysis is harnessing the energy stored in PEP in the form of ATP, a reaction catalyzed by pyruvate kinase (PK). In mammals, two PK genes give rise to four transcripts due to variations in splicing and all four isozymes have distinct biochemical and regulatory properties [183]. *PKLR* gene is differentially transcribed in liver and reticulocytes [184] to give PKL and PKR isozymes, respectively. In all other tissues, muscle-type PK (PKM) is expressed. Alternative splicing of *PKM* gene gives rise to PKM1 isozyme that is dominant in post-mitotic tissues while PKM2 is expressed during specific conditions [185]. As homotetramers, PKM catalyzes the oxidation of PEP to pyruvate and also generate an ATP molecule. PKM1 isoform strongly couples glycolysis to TCA cycle, while cancer cells use the PKM2 isoform to allow lactate buildup [186, 187]. However, PKM2 can exist as a dimer and appears to have protein kinase activity when ectopically expressed in cancer cell lines [188]. Here, the kinase activity of PKM2 [189] transferred the phosphate from PEP to many protein substrates assayed in vitro. Prominent substrates include STAT3 [190], MLC2, histone H3 [191], β -catenin [192] and ERK. Interestingly, one of the intermediates in purine biosynthesis was shown to activate the protein kinase function of PKM2 suggestive of a feed-forward mechanism [193]. PKM2, as a chaperone, activated the transcriptional function of HIF-1 α [194]. Whether the native PKM2 protein also behaves in the same manner is not known. Recently, non-canonical functions for enzymes of the glycolytic pathway (see [195] for a review) have been described viz., promoting cell migration, as a transcription factor, as a transcriptional co-activator etc. Since these studies were performed by over-expressed proteins in different cancer cell lines, the physiological and pathological relevance need to be evaluated further. PKM2 is the dominant isozyme during cellular proliferation and also expressed by cancer cells. In *Grim-19* deleted skin tumors, *Pkm2* levels go up compared to control tumors [118]. Thus due to a collective imbalance of handling glycolytic carbon, the intermediary products can be channeled for various catabolic and/or anabolic processes (Fig. 5). Proliferating cancer cells in culture convert pyruvate into lactate, catalyzed by lactate dehydrogenase (LDH) thereby regenerating NAD⁺. In mammals, three genes coding for *LDHA* (muscle-type), *LDHB* (heart-type) and *LDHC* (testis-type) are known [196]. The rest of the tissues express LDHA and LDHB to varying levels. The reaction catalyzed by LDHA favors the formation of lactate while LDHB favors the formation of pyruvate [197]. LDHA is overexpressed by numerous cancer types and cancer cell lines [198]. In *Grim-19* deleted primary MEFs, *Ldhd* transcript levels dropped more than 50% (Fig. 5). Thus Grim-19 loss may have indirectly promoted *Ldha* as the dominant isozyme in cells when measured for lactate levels [118]. A committed step of pyruvate channeling into TCA cycle is catalyzed by pyruvate dehydrogenase (PDH). PDH is a hetero-tetrameric enzyme [199] controlled by

four PDH kinases (PDK1, 2, 3 and 4) [200] and two PDH phosphatases (PDP1 and 2) [201]. Phosphorylation of a PDH subunit by PDKs inhibit while removal of the phosphate by PDPs activate PDH activity. *Pdk1* levels are increased in primary MEFs while *Pdk2* levels are increased in *Grim-19*-deficient skin tumors [118] (Fig. 5). In contrast *Pdk4* levels drop in *Grim-19* deleted skin tumors [118] compared to control tumors (Fig. 5). PDH complex is known to promote differentiation and/or induce senescence-like state [202]. Thus by lowering PDH activity, cancer cells can overcome senescence and replicate unhindered.

In a very simplified model of tumor growth, only an altered pH was necessary (see [203] for a review). The extracellular pH is normally more alkaline than the intracellular pH i.e., default pH. In tumors, the extracellular pH is more acidic than the intracellular pH i.e., reversed pH. This 'reversed pH gradient' was suggested to confer all advantages to tumor cells viz., proliferation, apoptosis evasion and enhanced migration. This reversal of pH also has consequences for efficacy and metabolism of anti-cancer drugs [204, 205]. In an acidic pH, drugs are unable to enter cells in the tumor microenvironment while normal cells become its unintended target causing toxic side effects. Even though lowered pH in the tumor microenvironment can prevent immune cells from attacking the tumor [206], an alternative model of metabolite competition was recently suggested to promote T cell hyporesponsiveness in the tumor [207]. As glucose is consumed by all cell types in the tumor, it appears that naïve T cells are unable to drive the rapid utilization of glucose i.e., aerobic glycolysis required for converting them to an activated T cell. This could be due to their inability to upregulate glucose and/or monocarboxylate transporters and compete with cancer cells that already have high levels of the same. Alternatively, excess lactate excreted by the cancer cells could contribute to the anti-proliferative and anergy of T cells. Interestingly, effector T cells and M1 macrophages that rely on glucose for their function are affected more than regulatory T cells and M2 macrophages that can sustain on alternate carbon sources (see [208] for a review). It is likely that competition for other nutrients is present in the tumor and the ability of immune cells to acquire them is also impaired when excess lactate is present.

Conclusions

Evolution of cancer involves multiple genetic and epigenetic alterations. Ten hallmark traits that confer an advantage for pre-cancerous cells to become clinically-relevant disease were recently postulated by Hanahan and Weinberg (Figure 6) [152]. Four of these traits viz., resisting cell death, evading growth suppression, maintaining sustained proliferative potential and attaining replicative immortality; do not directly hinder the function(s) of other non-cancerous cells *per se* in the tumor microenvironment. We know that GRIM-19 prevents three of these traits in cancer cell line models (Fig. 6). Two other traits viz., acquiring *de novo* mutations leading to rewired cellular energetics may be reliant on interactions with the tumor stroma. Wild-type GRIM-19 prevented the Warburg effect while mutant GRIM-19 could not prevent the same. The last four traits viz., tumor-promoting inflammation, invasive capacity, induction of neovasculature and avoiding immune destruction; do affect other cells in the tumor microenvironment. We and others have shown that GRIM-19 levels inversely correlated with metastatic spread in primary human tumors and blood vessel formation *in vivo* studies (Fig. 6). On similar lines, GRIM-19 point mutations were less efficient at

preventing cell migration *in vitro* and *in vivo* strongly suggests the importance of GRIM-19 vis-à-vis mitochondrial OXPHOS in preventing advanced disease.

GRIM-19 controls cell cycle arrest, induction of apoptosis and innate immune responses [92] while evidence for GRIM-19 controlling acquired immune responses [156, 209] is emerging. The role of mitochondrial proteins in immune response is poorly understood. With the identification of NLRX1 (a RC-3-interacting protein) to induce innate immune responses against viruses [210], the role of mitochondrial RC proteins to augment immune responses against pathogens are being increasingly investigated. Importantly, the ability of mitochondria to constantly sustain ROS generation to kill microbes and ROS detoxification to protect cellular contents are crucial for cell survival. The inability of aged male *Grim-19* heterozygous mice to mount immune responses against an opportunistic pathogen is a prime example for this [92]. Importantly, pharmacological inhibition of RC complexes prevented cytokine synthesis in bone marrow-derived macrophages. Curiously, it is unclear why female mice were not included in this study. Is this a sex-specific effect? The *XIAP* gene, mapping to X chromosome in human and mouse, could be a potential candidate that could influence the outcome. Mutations in *XIAP* were associated with reduced numbers of lymphocytes, the X-linked lymphoproliferative syndrome (XLP) [211]. Later the importance of XIAP to innate immunity was demonstrated by the total loss of Nod signaling in *Xiap*-deficient cells [212, 213]. In humans, many patients with IBD had mutations in *XIAP* gene and suffered from systemic inflammation. These individuals, however, did not show symptoms of XLP [214, 215]. Interestingly, our RNA-Seq analysis had revealed that upon *Grim-19* gene deletion, the most drastic changes occurred in genes involved in innate immune responses [118]. This raises an important question as to whether modulators of innate immune responses have partly overlapping roles in a cell/tissue-specific manner. Curiously enough, other IAPs are also intensely investigated for their role in innate immunity and inflammation (see [216] for a review). YM155, a small molecule inhibitor of Survivin [217] is being evaluated in clinical trials as an anti-cancer agent. The initial observation of mitochondrial Survivin [218] in cancer cell lines was recently shown to support mitochondrial repositioning, oxidative phosphorylation and invasion [73] using recombinant proteins. What role does GRIM-19 play in such a scenario remains to be examined.

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Fig. 1.

GRIM-19 controls cellular growth. GRIM-19 interacts with STAT3 TAD to block STAT3-driven transcription of genes involved in apoptosis resistance and cell proliferation. GRIM-19 interacts with p16Ink4A to strengthen the blockade on CDK4 imposed by p16Ink4A alone. Thus the activator, cyclin-D1, cannot promote G1-S transition as RB protein is not phosphorylated to relieve the inhibition on E2F1. GRIM-19 interacts with OLFM-4 to inhibit oxidative stress response genes during S phase of the cell cycle. Blue arrows represent stimulation of transcription. Red arrows represent biochemical events. Blunt red bars represent inhibition.

Fig. 2.

GRIM-19 induces degradation of oncoproteins. In high-risk HPV positive cancers, E6-E6AP complex promotes destruction of the p53 tumor suppressor and induces transcription of *TERT* gene. GRIM-19 interacts with E6 to prevent proteasomal destruction of p53. E6AP undergoes degradation upon binding GRIM-19. By preventing E6-E6AP complex formation, GRIM-19 maintains low levels of TERT protein. TERT activity is needed to maintain telomere length and for cancer cells to attain replicative immortality. Overexpression of XIAP is observed in many cancers. XIAP protein ubiquitylates pro-Caspase-9 that targets it for proteasomal destruction. GRIM-19 augments the proteolytic activity of HTRA2 to degrade XIAP and thus relieves the inhibition on pro-Caspase-9. Red arrows represent biochemical or signaling events.

Fig. 3.

Inducers and inhibitors of GRIM-19. GRIM-19 was discovered as an IFN-inducible gene product. Stimulation of NOD2, TLR2 and/or TLR4 upregulates *GRIM-19* transcription. The complete mechanism of transcriptional induction is not known currently. Viruses evade apoptosis of infected cells by multiple mechanisms. One such common mechanism adopted by viruses is to inhibit GRIM-19. Specific viral gene products employed are shown next to red bars. Blue arrows represent stimulation of transcription. Blunt red bars represent inhibition.

Fig. 4.

GRIM-19 prevents oncogene-induced migration. Proteins at focal adhesions are shown on the top left while the biochemical and transcriptional responses are shown on the left bottom. Right side shows the various cellular structures that can be differentiated under a light microscope. The mechanism of v-Src inhibition by GRIM-19 is shown in the central box. In the presence of wild-type GRIM-19, Pag1 levels are high that restricts the level of active v-Src. Blue arrows represent stimulation while blunt red bars represent inhibition.

Fig. 5.

Metabolic processes impacted by GRIM-19. Cellular GRIM-19 levels determine catabolic or anabolic fate of glucose. Major process affected due to GRIM-19 loss is shown in pink oval. Blue arrows represent processes activated in cancers. Most of these are derived from isotope tracing in cell culture models. Enzymes are shown inside rectangles. Enzymes affected due to GRIM-19 loss are shown in red font in yellow rectangles with a halo. Enzymes unaffected due to GRIM-19 loss are shown in grey rectangles. Double-headed arrow represents a reversible reaction while the arrow head size shows the direction of net flux in physiological conditions. Open black arrow represents stimulation and blunt red bars represent inhibition of activity. 1C denotes the 1-carbon transfer in nucleotide synthesis. GPD: Glycerol phosphate dehydrogenase, NNT: NAD(P) transhydrogenase. See text for additional details.

Fig. 6.

GRIM-19 blocks tumorigenesis at multiple levels. The ten hallmarks of cancer proposed by Hanahan and Weinberg is depicted. The top portion of the figure shows cancer cell characteristics independent of the microenvironment while the bottom portion shows characteristics that are influenced by other cell cells in the tumor. GRIM-19 prevents STAT3-driven cancer cell responses in the tumor microenvironment. The most important function of GRIM-19 is to fine tune cellular energetics leading to cancer cell adaptation in the tumor microenvironment. Blue arrows represent stimulatory responses, broken black arrows represent significant alterations in function and blunt red bars represent inhibition. See text for additional details.

Table 1

Expression level and mutational status of GRIM-19 in human cancers.

Tissue of tumor origin	Tumor (Normal)	Protein	RNA	DNA	Mutation(s)	Reference
Thyroid (Hurthle cell tumor)	5 (5)	-	-	5	K ⁵³ N, R ¹¹⁵ P	[122]
Kidney (RCC)	40 (40)	↓ IHC, WB	↓ qRT-PCR	-		[120]
	Splicing defect ^{LF}	-	↓ qRT-PCR	-		[219]
Prostate	17 (17)	↓ IHC	-	-		[120]
Prostate (Carcinoma)	Details not available	↓ IHC, WB	↓ qRT-PCR	-		[220]
Urinary bladder	6 (6)	NS	-	-		[120]
Gastro-intestinal	63 (63)	↓ IHC, WB	↓ qRT-PCR	-		[221]
Gastro-intestinal (Stomach)	32 (32)	↓ IHC, WB	↓ qRT-PCR	-		[222]
Gastro-intestinal (CRC)	39 (39)	↓ IHC	↓ qRT-PCR	-		[223]
Lung (NSCLC)	49 (49)	↓ IHC	-	-		[224]
Lung (ADC)	37 (37)	↓ WB	↓ qRT-PCR	-		[225]
	15	↓ IHC	-	-		[226]
Lung (SCLC)	10	↓ IHC	-	-		[226]
Lung (SCC)	16	↓ IHC	-	-		
Cervix (Carcinoma)	20 (15 [*])	↓ IHC, WB	↓ qRT-PCR	-		[72]
	60 (45 [*])	↓ WB	-	-		[99]
	Details not available	↓ IHC	-	-		[227]
Ovary	240 (46 [*])	↓ IHC, WB	-	-		[228]
Nervous system	34 (4 [*]) ^{LF}	-	↑ qRT-PCR	-		[229]
Nervous system (Gliomas)	58 (5)	↓ IHC, WB	↓ qRT-PCR	-		[230]
Liver (Carcinoma)	55 (55)	↓ IHC, WB	↓ qRT-PCR	-		[231]
Liver (Carcinoma)	83 (91)	-	↓ qRT-PCR	-		[232]
Liver (Carcinoma)	54 (54)	↓ IHC	-	-		[233]
Adrenal	14	↓ IHC, WB	-	-		[234]
Breast	128 (20)	↓ IHC, WB	-	-		[121]
Head & Neck (SCC)	12 (12)	-	↑ qRT-PCR	3	L ⁷¹ P, L ⁹¹ P, A ⁹⁵ T	[46]

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Tissue of tumor origin	Tumor (Normal)	Protein	RNA	DNA	Mutation(s)	Reference
HeLa cells		-	Yes	Yes	K ⁷ E (Heterozygous)	Nallar and Kalvakolanu (unpublished)
HSC2 and HSC3 cells		-	Yes	Yes	Y ¹⁴³ → Stop (Homo)	
HN-12 cells		-	Yes	Yes	D ⁹ E (Homo)	
Kidney		-	No	Yes	S ³¹ L, R ⁵⁷ C	COSMIC database ^{\$}
Lung		-	No	Yes	Y ³³ C, R ⁸¹ L	
Colon		-	No	Yes	R ¹¹⁵ C, G ¹³⁹ S	
Uterus (Endometrium)		-	No	Yes	T ¹⁴⁴ M	
Skin (Melanoma)		-	No	Yes	R ⁸² K	

* - unmatched tissue, LF - longer human GRIM-19 transcript,
\$ - 19,717 tumor DNA samples sequenced and mutations were heterozygous.