Ceramide Signaling and p53 Pathways

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Abstract

Ceramides, important players in signal transduction, interact with multiple cellular pathways, including p53 pathways. However, the relationship between ceramide and p53 is very complex, and mechanisms underlying their coregulation are diverse and not fully characterized. The role of p53, an important cellular regulator and a transcription factor, is linked to its tumor suppressor function. Ceramides are involved in the regulation of fundamental processes in cancer cells including cell death, proliferation, autophagy, and drug resistance. This regulation, however, can be pro-death or pro-survival depending on cancer type, the balance between ceramide species, the rate of their synthesis and utilization, and the availability of a specific array of downstream targets. This chapter highlights the central role of ceramide in sphingolipid metabolism, its role in cancer, specific effectors in ceramide pathways controlled by p53, and coregulation of ceramide and p53 signaling. We discuss the recent studies, which underscore the function of p53 in the regulation of ceramide pathways and the reciprocal regulation of p53 by ceramide. This complex relationship is based on several molecular mechanisms including the p53-dependent transcriptional regulation of enzymes in sphingolipid pathways, the activation of mutant p53 through ceramide-mediated alternative splicing, as well as modulation of the p53 function through direct and indirect effects on p53 coregulators and downstream targets. Further insight into the connections between ceramide and p53 will allow simultaneous targeting of the two pathways with a potential to yield more efficient anticancer therapeutics.

1. INTRODUCTION

Ceramides, members of the second largest class of membrane lipids, represent a family of more than 200 structurally related sphingolipids, compounds formed by a sphingoid base and a fatty acid linked via the amide bond (Hannun & Obeid, 2011, 2017). Over the past decades numerous studies have demonstrated that depending on their molecular structure, ceramides confer unique biophysical properties to the biological membranes (Castro, Prieto, & Silva, 2014). While the mechanisms of ceramide function in biological membranes and the biological consequences of ceramide alterations for cellular processes are still poorly understood, overwhelming data point to the role of these sphingolipids as important cellular regulators. Over the last 3 decades ceramides were established as second messengers regulating key cellular processes including cytoskeleton dynamics, endocytosis, protein

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transport and subcellular localization, cell cycle, autophagy, and apoptosis (Castro et al., 2014; Coant, Sakamoto, Mao, & Hannun, 2017; Dany & Ogretmen, 2015; Mullen, Hannun, & Obeid, 2012). Therefore, ceramides are indispensable for cellular homeostasis and control of fundamental functions, such as proliferation, migration, differentiation, adaptation to stress, survival, and senescence (Hannun & Obeid, 2008, 2017).

2.   CERAMIDE SIGNALING—BIOLOGY

Ceramide, a central molecule in sphingolipid metabolism (Hannun & Obeid, 2008; Levy & Futerman, 2010), can be generated by the de novo pathway, which starts from the serine and palmitoyl-CoA condensation. It can be also produced by the salvage pathway, whereby sphingolipids are hydrolyzed in the lysosomes to sphingosine, which can be reacylated back to ceramide by one of the ceramide synthases (CerSs) (Coant et al., 2017). Finally, it can originate from the direct hydrolysis of complex sphingolipids such as sphingomyelin and glycosphingolipids (Hannun & Obeid, 2017). Overall, ceramide biosynthesis and the downstream function are interconnected with other lipid pathways, highly compartmentalized within the cell and also engaged in communication with organelle-specific metabolic pathways (Coant et al., 2017; Hannun & Obeid, 2011; Hoeferlin, Fekry, Ogretmen, Krupenko, & Krupenko, 2013). Collectively, these aspects of ceramide metabolism call for a "Many Ceramides" approach (Hannun & Obeid, 2011) for investigating ceramide effects and their significance for biological processes. Such an approach requires characterization of the temporal and spatial dynamics of different ceramide species, identification of pathways regulating such dynamics, and the close look at specific cellular compartments where the changes take place. Understanding of the complex aspects of ceramide biology will provide a comprehensive picture of the ceramide function, allow more precise predictions of the end point effects of ceramide alterations, and enable the development of therapeutic means to modulate ceramide-dependent processes (Hannun & Obeid, 2011). Ultimately, not only ceramides but also sphingosine 1-phosphate (S1P), as well as related complex sphingolipids, determine the overall cellular response to different stimuli, a concept represented by a "sphingolipid rheostat" mechanism, originally suggested in 1998 (Mandala et al., 1998). Importantly, increased ceramide metabolism by certain enzymes such as glucosylceramide synthase (GCS), sphingomyelin synthase, ceramide kinase, ceramidases, and sphingosine kinases (SPHKs) has been shown for cancer cells, underscoring the role of sphingolipid metabolism in cancer. Toward this end, the increase in sphingolipids with prosurvival function emerged as a novel target for anticancer therapy (Ogretmen, 2018).

3.   CERAMIDE AND CANCER

Cancers are frequently characterized by altered levels of sphingolipids, including ceramide, but mechanisms by which these alterations result in tumor formation and progression are largely unknown. Ceramide-producing enzymes CerS2 (ceramide synthase 2) and CerS6 (ceramide synthase 6) were shown to be elevated in cancerous breast tissues as compared with normal breast tissues (Erez-Roman, Pienik, & Futerman, 2010). Endogenous C_{16}-, C_{24}-, and $C_{24:1}$-ceramide levels were increased in human head and neck squamous cell carcinomas, compared to normal tissues levels (Karahatay et al., 2007). Importantly, C_{16}-

and C_{18}-ceramides were demonstrated to play two opposing roles in human head and neck squamous cell carcinomas, prosurvival and proapoptotic, respectively (Senkal, Ponnusamy, Bielawski, Hannun, & Ogretmen, 2010). Over the years numerous studies presented evidence of the ceramide function in the signal transduction of cancer cells and in their response to nutrient and oxidative stress, chemotherapy, and radiation (Hannun & Obeid, 2017; Ogretmen & Hannun, 2004; Saddoughi et al., 2013). While initially ceramides were regarded as death-promoting signaling molecules, it is now believed that effect of the ceramide elevation depends on the specific ceramide structure and the cellular context (Mesicek et al., 2010; Schiffmann et al., 2009).

Ceramides also emerged as important biomarkers in cancer therapy. For example, increase of total plasma ceramide in the patients undergoing radiation therapy in combination with irinotecan chemotherapy was associated with favorable tumor response (Dubois et al., 2016). Analogously, CerS6 was identified as a member of the 19-gene-signature predicting survival of patients with colorectal cancer (Abdul Aziz et al., 2016). Mechanistic basis for the distinct functions of endogenous ceramides is not known; however, interaction of ceramides with specific proteins was suggested to be responsible for this, at least in part (Saddoughi et al., 2013).

Another important aspect of altered ceramide levels in cancer, specific elevation of ceramide-metabolizing enzymes, is connected to cancer resistance, a major obstacle in cancer therapy. Thus, elevation of SPHK1 and GCS in prostate cancer cells was found to be responsible for resistance to paclitaxel (Aoyama et al., 2017). Elevated GCS expression has been demonstrated to correlate with progression of breast, urinary, ovarian cancers, and leukemia (Liu, Hill, & Li, 2013), whereas upregulation of acid ceramidase on irradiation conferred prostate cancer resistance (Cheng et al., 2013). This opens an opportunity to target these enzymes for treatment of resistant tumors (Ogretmen, 2018).

4. P53: THE BEGINNING

The p53 transcription factor, originally discovered as an endogenous cellular protein bound to a simian virus 40 large T antigen (Lane & Crawford, 1979; Linzer & Levine, 1979), has been initially described as an oncogene. Later studies demonstrated that a normal p53 gene is not required for embryonic development, but its absence predisposes mice to neoplastic disease, and that the oncogenic mutant forms of p53 are not required for development of many types of cancer (Donehower et al., 1992). Thus, the p53 protein was established as tumor suppressor. It is also commonly referred to as the guardian of the genome, due to its strong ability to respond to DNA damage or to stress stimuli capable of the induction of such damage (Lane, 1992). *TP53*, the gene encoding for the p53 protein, is the most often mutated gene in human cancers with the mutation rate estimated to be above 50% across all cancer types (Shirole et al., 2016). Due to the fact that p53 protein does not easily respond to pharmacological intervention, it has been considered "undruggable," and therapies capable of overcoming the oncogenic effects of mutant p53 have not been developed yet, almost 40 years after discovery of the protein function in cancer (Sabapathy & Lane, 2018). Nevertheless, the line of research evaluating p53 as a chemotherapeutic target tremendously expanded our understanding of the protein structure and function.

5.   P53 IS A TRANSCRIPTION FACTOR

Regardless of all the nicknames of the p53 protein, first and foremost, it is a transcription factor controlling expression of hundreds of targets (Fischer, 2017). At the transcriptional level, p53 acts exclusively as a direct transcriptional activator, whereas inhibitory effects on some targets are usually indirect and mediated by cyclin-dependent kinase inhibitor p21 (CDKN1A or Waf1) interfering with phosphorylation of the retinoblastoma-like proteins RBL1 (p107) and RBL2 (p130) (Quaas, Muller, & Engeland, 2012; Sullivan, Galbraith, Andrysik, & Espinosa, 2018). In turn, hypophosphorylated RBLs stabilize a multicomponent repressor DREAM (including dimerization proteins, RBLs, E2F4, and multivulval class B complex [MUVB]), which inhibits transcription by engaging E2F or cell cycle homology region sites and suppresses transcription (Fischer, 2017). In addition, p53 may indirectly suppress transcription by a variety of mechanisms, such as binding to and disabling specific transcription factors, outcompeting specific activators from promoters due to overlapping binding sites, recruiting chromatin-modifying complexes that block gene expression, and via regulation of both miRNAs and lincRNAs (Barsotti & Prives, 20101; Huarte et al., 2010).

The p53 protein is a highly labile and tightly controlled transcription factor present in nonstressed cells at low level due to its binding by E3 ubiquitin ligase MDM2, which enables p53 ubiquitination and proteasomal degradation (Hock & Vousden, 2014). On a variety of cellular cues, p53 escapes the control by MDM2, accumulates in the nuclei, and activates multiple genes to regulate diverse cellular processes including progression through the cell cycle, DNA damage repair, apoptosis, metabolic alterations, epigenomic modifications, shortening of telomeres, ferroptosis, etc (Haupt & Haupt, 2017). While numerous data indicate that p53 can regulate a wide array of biological processes, it should be noted that cell type, context, and specific stimuli define not just the different levels of gene expression but rather affect the transcription of very different sets of genes (Kastenhuber & Lowe, 2017). Thus, it is not surprising that depending on the cell type, p53 can generate opposite responses to the same stressor. Still, in many cases, mechanisms for such differences are not known. It has been suggested that context-dependent posttranslational modifications of p53 may dictate the interaction with a specific gene target (Kumari, Kohli, & Das, 2014). In addition, the kinetics of the p53 activation and the architecture of core promoters play a role in defining cellular response in a specific case: short p53 activation with rapid assembly of preinitiation complex and few rounds of RNA polymerase II reinitiation taking place at the p21 core promoter, and sustained activation with slow assembly of preinitiation complex that supports multiple rounds of transcription in case of Fas/APO1 and PUMA (Gomes & Espinosa, 2010; Morachis, Murawsky, & Emerson, 2010). The presence of epigenetic marks and other transcription factors at the target promoters may further attenuate the gene response to p53 activation (Itahana et al., 2016; Kastenhuber & Lowe, 2017). Thus, p53 functions in a complex network of regulators that support flexibility of cellular response to changing environment, depending on the cell type and state.

6. P53 AS A METABOLIC REGULATOR

Despite the canonical p53 definition as a tumor suppressor, one should keep in mind that ancestral members of the p53 gene family, found in unicellular protists and short-lived multicellular organisms (*Drosophila*) that do not develop cancers, function only in germ stem cells as protectors from DNA damage and uncontrolled proliferation (in the absence of DNA damage) (Wylie, Lu, D'Brot, Buszczak, & Abrams, 2014). These findings point to the cancer prevention by p53 as a later development of an ancient ability to prevent DNA damage and regulate growth of stem cells. It is also believed that the p53 regulatory network acquired diverse physiological roles before it was engaged in tumor suppression (Kastenhuber & Lowe, 2017). A few examples demonstrating the breadth of the p53 regulatory network include the response to various types of cellular stress, such as DNA damage (Williams & Schumacher, 2016), nutrient deprivation (Hoeferlin et al., 2013), hypoxia (Humpton & Vousden, 2016), and ribosomal stress (Deisenroth, Franklin, & Zhang, 2016). In addition, p53 regulates metabolic activities of the cell (Floter, Kaymak, & Schulze, 2017), the epigenome (Haupt & Haupt, 2017), and miRNAs (Liu, Zhang, Zhao, & Feng, 2017). Importantly, ceramide elevation is also observed in response to DNA damage, nutrient stress, senescence, autophagy, and apoptosis (Hannun & Obeid, 2017), pointing to a possible connection between the two pathways.

As a transcription factor, p53 regulates a variety of metabolic pathways, including glycolysis, pentose phosphate pathway, serine metabolism, mitochondrial metabolism, redox status, and lipid metabolism (Floter et al., 2017). By limiting NADPH production, p53 slows down many anabolic processes, including fatty acid biosynthesis. At the same time, wild-type (WT) p53 upregulates fatty acid oxidation by elevating expression of lipin 1 and guanidinoacetate methyltransferase (Liu, Zhang, Hu, & Feng, 2015). Moreover, via direct transcriptional control of such targets as sterol-regulatory-binding protein-1 (SREBP-1), acetyl-CoA dehydrogenase family member 11 (ACAD11), LIPIN1, malonyl-CoA decarboxylase (MCD), dehydrogenase/reductase 3 (DHRS3), carnitine palmitoyltransferase 1C (CPT1C) and caveolin 1, p53 controls multiple nodes in lipid metabolism (Parrales & Iwakuma, 2016).

Additional role of p53, different from the transcription activation, is known (Green & Kroemer, 2009; Vaseva & Moll, 2009). It is based on the ability of p53 to interact with myriads of cellular proteins thus affecting their functions. Importantly, this nontransactivation function of p53 can be fulfilled by WT protein and by its mutant variants. For example, while WT p53 suppresses the SREBP-1 levels via yet unidentified mechanisms, the R273H and R280K p53 mutants can bind to SREBP increasing its activity. Because p53 is frequently mutated in cancers, this further expands the processes controlled by p53. Another example includes the binding of mutant p53 to AMPK that inhibits this kinase activity (Zhou et al., 2014). The consequences of this effect can be profound taking into consideration that AMPK has recently emerged as a key regulator of cellular metabolism. Finally, it should be mentioned that p53 has yet another function associated with its translocation to mitochondria. This pathway involves the p53 interaction with members of the Bcl2 family and regulates apoptosis (Marchenko & Moll, 2014; Vaseva & Moll, 2009).

7. P53 IN TUMOR SUPPRESSION

Since the discovery of a tumor suppressor function of p53, it was believed to exert its cancer suppressor role by inducing senescence and apoptosis. Later it was demonstrated that p53 proficient mice, contrary to mice deficient in this protein, were completely suppressing development of thymic lymphomas even in the absence of p21, PUMA, and NOXA, critical players in p53-dependent senescence and programmed cell death (Valente et al., 2013). Moreover, mice expressing the p53^{3KR} mutant, deficient in apoptotic and growth arrest functions but proficient in noncanonical and metabolic-regulatory functions, were still protected from tumor formation (Wang et al., 2016). These studies underscore the significance of the p53-mediated transcription for tumor suppression. However, identification of the p53 target genes and responses contributing to tumor suppression is very challenging, and only a few of already identified mechanisms have been tested in vivo (Kaiser & Attardi, 2018).

8. MUTANT P53 FUNCTION

The variety and frequency of p53 mutations in cancer deserve special consideration. Simplistically, the 393-amino-acid protein is organized in several functional domains, the amino terminal transactivation domain (residues 1–92), the DNA-binding domain (DBD, residues 101–306), the oligomerization domain (residues 307–355), and the carboxy-terminal domain (residues 356–393). Of note, tetramerization of p53 is crucial for its activity as a transcription factor (Lomax, Barnes, Hupp, Picksley, & Camplejohn, 1998). While the high rate of the *TP53* mutations affecting more than 300 amino acid residues creates an impediment for development of the p53-targeting therapeutics, a novel paradigm of "a rainbow of mutants" may lead to the development of efficient, mutant type—specific therapeutics (Sabapathy & Lane, 2018). The idea of the p53 mutants possessing functional differences suggests differential approach for targeting tumors with specific types of the protein mutations. Thus, mutations in the amino terminal domain often result in the utilization of an alternative start codon and expression of a shorter version of the protein, p47, defective in induction of the cell cycle arrest, but retaining selective transactivation function and ability to induce apoptotic genes (Phang et al., 2015). These mutations predispose organisms to cancer but predict better survival on therapeutic intervention. Distinct from the amino terminal domain, mutations in oligomerization domain result in the loss of function by p53 proteins, inability to form homotetramers, limited or absent transactivation capability, and absent tumor suppressor activity (Giacomazzi et al., 2014). Importantly, approximately 85% of all *TP53* mutations are found in the central 205-amino-acid DBD (Bouaoun et al., 2016). Most of the p53 DBD mutations lead to the loss of the ability to activate genes targeted by nonmutated p53 (Lee et al., 2012). Indeed, the rate of tumorigenesis is similar in *TP53$^{-/-}$* mice and mice homozygous for mutant *TP53*, confirming the loss of tumor suppression function by the mutant. Interestingly, the dominant-negative effect of the p53 mutations makes WT allele dysfunctional when the cells are stressed by chemotherapeutics or irradiation (Lee et al., 2012; Willis, Jung, Wakefield, & Chen, 2004). So, an approach to downregulate only mutant p53 keeps promise for improvement of the tumor sensitivity to cancer drugs.

In the past decades numerous studies confirmed the "gain of function" hypothesis, which proposed that some DBD mutants may not just have lost the WT p53 activity but rather acquired an oncogenic activity (gained function), independent of their effects on the WT protein (Freed-Pastor & Prives, 2012). The addiction of tumors to mutant p53 was demonstrated in the mutant *TP53* knock-in mice, as the tamoxifen-induced mutant p53 ablation resulted in reduction of tumor growth, induction of apoptosis, and tumor regression, leading to 37% increase in animal survival (Alexandrova et al., 2015). It is believed that "gain of function" characteristics of the mutant p53 could be achieved via one of the two major mechanisms: a mutation conferring ability to transactivate the completely new set of genes (possibly via interaction with other transcription factors and chromatin remodeling proteins) and/or the interaction with other cellular proteins/signaling pathways (Freed-Pastor & Prives, 2012; Kim & Lozano, 2018). Precise details of both of these mechanisms will be dictated by the cell type and context and may be rather dynamic. Not all but only some of the p53 mutants display "gain of function" properties, and these properties seem to manifest in cancer cells only (Sabapathy & Lane, 2018).

Thus, accumulating evidence points to a potential benefit of therapeutic approaches targeting p53, as long as they take into account the cancer type, p53 status, and cellular context.

9. P53 AND CERAMIDE PATHWAYS

Cancer research in the past decades consistently implicated both the p53 and ceramide pathways in the regulation of cell growth, cell cycle arrest, senescence, and apoptosis. Therefore, it is not surprising that the connection between the two has been regularly reviewed (Carroll, Donaldson, & Obeid, 2015; Hage-Sleiman, Esmerian, Kobeissy, & Dbaibo, 2013; Heffernan-Stroud & Obeid, 2011).

Even the first study, which discovered a dose- and time-dependent elevation of the endogenous ceramide following the p53 activation by actinomycin D or gamma irradiation, observed a complex relationship between p53 and ceramide. The authors concluded that both p53-dependent and p53-independent stress response pathways could be activated by DNA damage, and ceramide accumulation could be a common feature in both of them (Dbaibo et al., 1998). Since then, numerous reports have demonstrated ceramide function both, downstream of p53 activation, upstream of p53, or independent of p53, in different cell types and under different stimuli (Heffernan-Stroud & Obeid, 2011). Interestingly, in *Caenorhabditis elegans*, radiation-induced apoptosis of germ cells was induced by the parallel pathways of CEP-1 (worm homologue of p53)-mediated elevation of the BH3-domain protein EGL-1, and CerS-mediated ceramide accumulation that converged at the mitochondrial membrane (Deng et al., 2008), thus representing yet another, synergistic, relationship between the p53 and ceramide.

Several sphingolipid-metabolizing enzymes, which produce or degrade ceramide, are regulated by p53 (Fig. 1). Thus, activation of the DNA damage pathway by doxorubicin resulted in the increase of neutral sphingomyelinase 2 (nSMase 2) activity and elevation of ceramide levels, as well as in induction of cell growth arrest. Activation of the nSMase was a result of direct transcriptional activation by p53 at a novel start site upstream of exon 3

(Shamseddine et al., 2015). In contrast, there is practically no evidence on acid sphingomyelinase (aSMase) activation by p53. In one of the recent studies, a derivative of PRIMA-1 (p53 reactivation and induction of massive apoptosis, compound 8b) was found to activate aSMase in H1299 cells (null for p53). PRIMA-1 is a synthetic compound that forms adducts with the p53 thiol groups and facilitates proper protein folding, thus correcting effects of p53-disabling mutations (Soans, Evans, Cipolla, & Fernandes, 2014). However, when the activated targets in H1299 $p53^{+/+}$ and H1299 cells were compared, this led to a conclusion that the compound demonstrated an off-target effect and activated the TNF receptor superfamily pathway in these cells, arguing against p53 involvement. In a different study, p53 was shown to activate the nSMase but not aSMase (Sawada et al., 2002). Studies in different cell lines, leukemic and colon carcinoma, revealed that, on p53 upregulation, the de novo pathway is a major contributor to ceramide accumulation. Interestingly, the increase in ceramide was associated with the transcriptional upregulation of CerS5 (ceramide synthase 5) and not with change in serine palmitoyltransferase (Panjarian et al., 2008).

Another ceramide synthase, CerS6, was recently found to be directly transcriptionally activated by p53 via a noncanonical binding site, 11 nucleotides upstream of transcription start site (Fekry, Jeffries, et al., 2016). In contrast to the upregulation of ceramide synthases, SPHK1, a sphingolipid enzyme balancing the ceramide/S1P rheostat (Baran et al., 2007), was shown to be downregulated by p53 (Heffernan-Stroud et al., 2012; Taha et al., 2004). This regulation involves both canonical effector caspases of the mitochondrial apoptotic pathway and other, noncaspase/lysosomal proteases, such as cathepsins. More recent data revealed that p53-mediated activation of caspase-2 was required for SPHK1 proteolysis. In the triple-negative breast cancer cells with mutated p53, caspase-2 was not activated by doxorubicin treatment, and SPHK1 activity was preserved. However, suppression of the CHK1 was able to activate both caspase-2 and SPHK1 degradation (Carroll, Bonica, Shamseddine, Hannun, & Obeid, 2018).

Overall, the information regarding the regulatory effects of p53 on sphingolipid enzymes and the mechanisms involved in this regulation is rather scarce.

10. ACTIVATION OF P53 BY CERAMIDE

The relationship between ceramide and p53 is often interrogated by treatment of cells with exogenous ceramides. Because natural ceramides are highly hydrophobic and are not soluble in aqueous media, either short-chain analogs (C_2- or C_6-ceramides) or soluble derivatives of the ceramide carrying pyridinium group have been used in these studies (Hou et al., 2011). For example, C_2-ceramide was shown to induce SKN-SH cell death via elevation of p53, subsequent increase in Bax/Bcl-2 ratio and caspase activation (Kim et al., 2002). Similarly, in murine myeloid NSF.H7 cells, C_2-ceramide activated the protein phosphatase 2A (PP2A) with a consequent dephosphorylation of the Bcl-2, that allowed for direct binding of Bcl-2 and p53, thus preventing Bcl-2 from disabling the proapoptotic Bad and Bax proteins and leading to apoptosis (Deng, Gao, & May, 2009). In this case, p53 function was not dependent on ceramide but contributed to its downstream mechanisms. On the other hand, treatment of HCT116 adenocarcinoma cells with exogenous C_{16}-ceramide upregulated expression of p53 and, additionally, modulated expression of 50 cellular proteins (Renert et

al., 2009). Experiments in which mouse fibroblasts were treated by alkylating agent MNNG (1-methyl-3-nitro-1-nitrosoguanidine) demonstrated that DNA damage response triggered in these cells was mediated by ceramide independently of p53 (Yang & Duerksen-Hughes, 2001). Nevertheless, the authors concluded that although the pathways were separable, they were interacting with each other, as the effects of ceramide on p53 levels and stability were noted. Another study concluded, based on the differences in morphology and DNA staining, as well as on the timing of the apoptotic death, that radiation-induced apoptosis was p53 dependent and that ceramide-induced apoptosis was not (Shi, Wuergler, Blattmann, & Crompton, 2001). Furthermore, a complex relationship between p53 and ceramide was also found on investigation of the cellular response to folate stress (Hoeferlin et al., 2013). Stressing A549 cells by either depletion of folate from culture media or by ectopic expression of a major folate-regulatory enzyme caused p53-dependent activation of de novo ceramide biosynthesis and CerS6-dependent C_{16}-ceramide elevation followed by apoptosis. Furthermore, overexpression of CerS6 by transient transfection, without any additional stress, resulted in strong p53 elevation and apoptosis, suggesting a feedback mechanism between C_{16}-ceramide and the tumor suppressor (Fig. 1).

Connection between p53 and ceramide pathways is often exploited in search for the combinational therapeutics. Coadministration of C_6-ceramide with chemotherapeutic drug vincristine caused necrosis and apoptosis in multiple cell lines. Significant activation of AMPK followed by p53 activation, inactivation of mTORC1, and downregulation of Bcl-1/ Hif-1α were observed in these cells. Activated p53 translocated to mitochondria and bound to cyclophilin D, which led to mitochondrial permeability pore opening and cell necrosis. The authors suggested that p53 activation by these synergistic drugs activates both necroptosis and apoptosis (Chen et al., 2015). Although the number of studies demonstrating ceramide effects on p53 is steadily increasing, our knowledge of the mechanisms of ceramide effects on p53 levels and activity is still rudimentary.

11. ACTIVATION OF OTHER GENES BY CERAMIDE

Ceramide has been reported to regulate expression of several genes. Early investigations of the ceramide function identified several genes regulated by ceramide, such as cyclooxygenase (Ballou, Chao, Holness, Barker, & Raghow, 1992) and IL-6 (Laulederkind, Bielawska, Raghow, Hannun, & Ballou, 1995). MMP-1 expression was shown to be induced by endogenous or short-chain exogenous ceramides via activation of three distinct MAPK pathways in fibroblasts (Reunanen et al., 1998). Ceramide treatment of human hepatocarcinoma cells induced p53-independent elevation of the p21 mRNA and protein, as well as G1 cell cycle arrest (Kim, Kang, Kim, & Choi, 2000). Furthermore, both exogenous and endogenous ceramides were shown to reduce the hTERT (human telomerase reverse transcriptase) promoter activity (Wooten & Ogretmen, 2005). The mechanism of TERT inhibition involved reduction of acetylation of the Sp3 transcription factor, increasing the ratio of Sp3/Sp1 occupying the promoter and reducing the recruitment of RNA polymerase II to the promoter. Interestingly, a sphingolipid enzyme GCS implicated in the development of resistance to cancer chemotherapy was found to be transcriptionally upregulated by the ceramide, with the Sp1 transcription factor being essential for this regulation (Liu et al., 2008). In a different study, cannabinoid treatment—induced de novo ceramide generation

activated transcription and increased protein levels of the stress-regulated protein 8 (p8) and its downstream targets Atf-4 and TRB3, leading to apoptosis of the pancreatic cancer cells (Carracedo et al., 2006). As in the case of p53, the mechanisms by which ceramide affects expression of its numerous targets are not fully understood.

12. CERAMIDE SIGNALING: MECHANISMS

Over the years, extensive research presented compelling evidence that changes in cellular ceramide levels are followed by activation of downstream effectors, which results in cell cycle arrest and adaptation, senescence, or programmed cell death.

Because ceramide is a hydrophobic molecule synthesized in and localized to the membranes, one of the plausible mechanisms of its effects on the downstream signaling molecules could be the changes in the biophysical properties of the membranes (lipid rafts formation, fluidity, etc.) where the changes in ceramide occur. These changes affect both the arrangement and properties of the membrane proteins, such as receptors, transporters, and signaling complexes that will transmit the signal further down the line (Edmond et al., 2015; Lockshon et al., 2012). In addition, both short- and long-chain ceramides were able to form stable ceramide channels in planar membranes in vitro, though the experimental evidence for this process in vivo is still missing (Hernandez-Corbacho, Salama, Canals, Senkal, & Obeid, 2017; Siskind & Colombini, 2000). Alternatively, ceramide elevation resulting from treatment of A549 cells with methotrexate caused ER (endoplasmic reticulum) membrane aggregation leading to apoptosis (Fekry, Esmaeilniakooshkghazi, Krupenko, & Krupenko, 2016).

Another mechanism could be the direct binding of ceramide to the components of a signaling pathway that changes their activity. Direct binding of ceramide has been shown for protein kinases PKCα, PKCδ, PKCz, KSR, and c-Raf. PKCα was shown to be activated on ceramide binding, and its translocation from cytosol to the membrane was delayed, whereas PKCδ showed no change in activity on binding but rather decreased autophosphorylation (Huwiler, Fabbro, & Pfeilschifter, 1998). PKCz interaction with ceramide via the cysteine-rich domain was found to elevate its activity and induce the interaction with stress-activated protein kinase (SAPK) complex, resulting in growth suppression (Bourbon, Yun, & Kester, 2000). Similarly, both endogenous and exogenous ceramides were able to bind to the KSR cysteine-rich domain C1 and activate it via autophosphorylation that promoted binding to and transactivation of Raf-1 (Zhang et al., 1997) (Yin et al., 2009). Curiously, using the photoactivatable iodine-labeled ceramide, c-Raf itself was identified as a ceramide-binding protein, which was activated on ligand binding and further activated MAPK1 and MAPK3 signaling cascade (Huwiler et al., 1996). Importantly, the regulation by direct physical binding to its target is not limited to ceramide. Recently, S1P was demonstrated to function as a cofactor of TRAF2 (TNF receptor—associated factor 2), which, on binding, stimulated the ubiquitin-ligase activity of the protein toward RIP1 (Alvarez et al., 2010).

Recent searches for ceramide-binding proteins using bifunctional lipids with a photoactivatable diazirine group as a bait revealed 20 candidates among cellular cytosolic proteins that were cross-linked to the labeled ceramide (Bockelmann et al., 2018). Identified

targets are involved in a variety of cellular functions, including ceramide transport, DNA damage response (DNA damage binding protein 1, and DnaJ homologue DnaJc7), protein ubiquitination (COP9 signalosome subunit 3), protein sorting (sorting nexin-1 and 2), trafficking (unc-119 A and B), and metabolism (GLO1, ALDH7A1, ADO, PTGR2, HMGCS2, AKR1C13, CTPS1, PAOX). This underscores the broad spectrum of processes regulated by ceramide (Bockelmann et al., 2018). However, none of the ceramide-metabolizing enzymes have been identified in this work; this is likely due to exclusion of all cellular membranes from the experiments.

Another plausible mechanism for regulation of gene expression by ceramide could be the activation of protein phosphatase 1 (PP1) and PP2A by ceramide. While PP1 was shown to bind ceramide on its own both in vitro and in vivo (Sumanasekera et al., 2012), the activation of PP2A was mediated by the direct binding of ceramide to the inhibitor of PP2A (IPP2A/SET (SET nuclear protoco-oncogene)), as demonstrated by pull-down of SET on biotinylated C_6-ceramide and avidin column (Mukhopadhyay et al., 2009). Moreover, specificity toward C_{18}-ceramide, and not to C_{16}-ceramide, was described for the PP2A inhibitor. Ceramide binding by PP1 inhibited the enzyme, whereas binding of ceramide by SET resulted in activation of PP2A accompanied by proteasomal degradation of c-Myc and tumor suppression (Mukhopadhyay et al., 2009; Oaks & Ogretmen, 2014). Change in the activity of either major cellular phosphatase may also affect phosphorylation status of multiple targets, such as transcription factors, signaling proteins, etc., and contribute to regulation of gene activity as well.

In line with these findings, it has been shown that in HEK293 cells, natural C_{16}- and C_{24}-ceramides bind and inhibit PP1, thus preserving phosphorylation of a group of splicing-regulatory proteins containing the evolutionarily conserved motif RVxF recognized by PP1 (Sumanasekera et al., 2012). On the other hand, in multidrug resistant NCI/ADR-RES ovarian cancer cells expressing mutant p53 (five-amino-acid deletion in exon 5), the synthetic pyridinium C_6-ceramide activated PP1 thus causing dephosphorylation of serine/arginine-rich splicing-factor 1 and inducing its translocation to the nucleus (Patwardhan et al., 2014). This enabled the alternative splicing of the pre-mRNA encoding the p53 deletion mutant and restoration of the mRNA splice variant translated into WT p53. In a similar manner, recovery of the WT p53 mRNA expression on GCS knockdown and ceramide accumulation in OVCAR-8 and NCI/ADR-RES cells (both heterozygous for the p53 exon 5 deletion) led to the elevation of the p53 transcriptional targets p21, Bax, and PUMA, as well as induction of apoptosis (Liu et al., 2011). The same group has also reported that the inhibition of GCS, accompanied by the elevation of intracellular ceramide, restored the WT p53 activity in the cancer cells heterozygous for a missense R273H mutant. This further sensitized cells toward doxorubicin and increased apoptosis (Hosain et al., 2016). Ceramide-mediated alternative splicing by the function of the splice factor SRp30a was also shown for Bcl-x and caspase 9 (Chalfant et al., 2002; Massiello & Chalfant, 2006).

Thus the number of ceramide-binding protein targets is increasing as we learn more about the signaling functions of ceramide, yet we still do not understand how ceramide is delivered to specific targets (especially to cytosolic proteins), how specificity of the binding is

achieved, and what mechanisms modulate the targets activity. The downstream effectors of ceramide-binding proteins activated/inhibited by ceramide are also often unknown.

13. CONCLUSIONS

Ceramides emerged as important novel signaling entities in the early 90s (Hannun, Obeid, & Wolff, 1993), and the past 25 years of research have revealed significant structural variability of ceramides, their involvement in vast array of biological processes, as well as very complex and intertwined relationships with their targets. An important role for ceramide in cancer development and response to therapeutic interventions has been also established, although our understanding of precise mechanisms guiding the cell type—specific and context-depending responses generated by ceramide alterations is still limited.

Tumor suppressor protein p53, discovered about 15 years earlier, is now, perhaps, the most studied protein, with the number of publications considering it approaching 90,000. These studies revealed many details about the protein structure, regulation, biological function, and its critical role for cancer suppression and therapy. Interestingly, similar to ceramide, the mechanisms of the p53 function are also cell type—specific and stimulus-specific, and highly context-dependent. Despite the years of intensive research, our understanding of these mechanisms is still incomplete.

In recent years, numerous studies indicated connections between the ceramide and p53 pathways (Fig. 1). Indeed, both of the pathways have been implicated in the critical cell fate decision processes, such as cell cycle arrest, senescence, and apoptosis. In these processes, ceramide and p53 can function upstream or downstream of each other, making the overall relationship quite complex. In this regard, due to cell type—dependent and context- and stimulus-dependent nature of both pathways, their intersection increases the variability of the response exponentially, making the repertoire of adaptive changes significantly larger. Accordingly, the task of examining these adaptations is extremely complex. Further investigation of the sphingolipid enzyme structures and regulation, identification of ceramide-specific protein targets, and changes that ceramide binding confers, are necessary to significantly improve our ability to untangle the mixed and sometimes contradictory data regarding the connection between p53 and ceramide. As our knowledge of both the ceramide and p53 function expands, it will reveal the regulatory nodes that can be efficiently targeted for therapeutic intervention in cancers.

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(A) **Effect of p53 mediated by ceramide pathways**
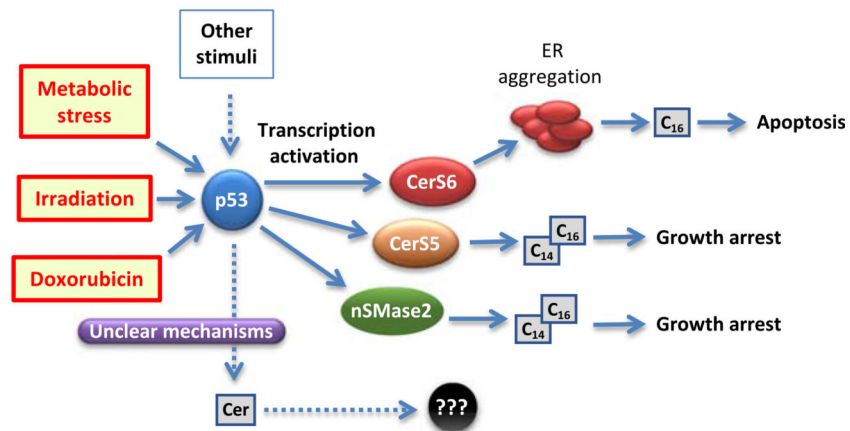
(B) **Regulation of p53 by ceramide**

Figure 1. Circuits connecting the p53 function and ceramide signaling.
(A) The induction of p53 has been associated with transcriptional activation of several enzymes of ceramide metabolism including CerS6 (in response to folate-mediated metabolic stress); CerS5 (in response to γ-irradiation); and nSMase (in response to doxorubicin). In all cases, C_{14}- and/or C_{16}-ceramides accumulated that caused antiproliferative effects (growth arrest or apoptosis). Other stress stimuli, which induce p53, can cause accumulation of certain ceramides, thus implicating p53 in this outcome, though precise underlying mechanisms remain unclear. (B). As part of signal transduction, ceramides themselves could activate p53 as a downstream target. Such effect has been demonstrated for C_2- and C_{16}-ceramides as well as for CerS6 (perhaps through the C_{16} generation as an intermediate step).