Native and polyubiquitinated forms of dihydroceramide desaturase are differentially linked to human embryonic kidney cell survival

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Abbreviations: ABC294640, 3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide; CerS, ceramide synthase; CHOP, CCAAT-enhancer-binding protein homologous protein; Degs1, dihydroceramide desaturase; ER, endoplasmic reticulum; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; MDM2, human homologue of Mouse Double Minute 2; MG132, carbobenzoxy-Leu-Leu-leucinal; NAC, N-acetyl-L-cysteine; PARP, Poly(ADP-Ribose) Polymerase; PERK, protein kinase R-like ER kinase; S1P, sphingosine 1-phosphate; SK1, sphingosine kinase 1; SK2, sphingosine kinase 2; SKi, 2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole; UPR, unfolded protein response; XBP-1s, X-box protein-1s.

ABSTRACT--There is controversy concerning the role of dihydroceramide desaturase (Degs1) in regulating cell survival with studies showing that it can both promote and protect against apoptosis. We have therefore, investigated the molecular basis for these opposing roles of Degs1. Treatment of HEK293T cells with the sphingosine kinase inhibitor, SKi (2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole)) or fenretinide, but not the Degs1 inhibitor, GT11 (((N-[(1R,2S)-2-hydroxy-1-hydroxymethyl-2-(2-tridecyl-1-cyclopropenyl)ethyl]octan-amide)) induced the polyubiquitination of Degs1 (Mr=40-140 kDa) via a mechanism involving oxidative stress, p38 MAPK and Mdm2 (E3 ligase). The polyubiquitinated forms of Degs1 exhibit 'gain of function' and activate pro-survival pathways, p38 MAPK, JNK and X-box protein-1s (XBP-1s). In contrast, another sphingosine kinase inhibitor, ABC294640 (3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide) at concentrations of 25-50 μM failed to induce formation of the polyubiquitinated forms of Degs1. In contrast with SKi, ABC294640 (25 μM) promotes apoptosis of HEK293T cells via a Degs1-dependent mechanism that is associated with increased *de novo* synthesis of ceramide. These findings are the first to demonstrate that the polyubiquitination of Degs1 appears to change its function from pro-apoptotic to pro-survival. Thus, polyubiquitination of Degs1 might provide an explanation for the reported opposing functions of this enzyme on cell survival/apoptosis.

Introduction

De novo synthesis of ceramide involves a pathway in which serine and palmitoyl CoA undergo condensation to form 3-ketosphinganine, catalysed by serine palmitoyltransferase and which is the rate limiting step in ceramide biosynthesis (1). 3-Ketosphinganine is converted into sphinganine (dihydrosphingosine) by 3-ketosphinganine reductase, which is then acylated to dihydroceramide by ceramide synthases (CerS). Dihydroceramide desaturase (Degs1) introduces a *trans* 4,5 double bond to dihydroceramide, thereby forming ceramide *de novo*. Ceramide can also be formed by the sphingomyelinase-catalysed hydrolysis of sphingomyelin in the so-called 'salvage' pathway. Deacylation of ceramide by ceramidase produces sphingosine (Sph), which is then phosphorylated by sphingosine kinase 1 or 2 (SK1 or SK2) to form sphingosine 1-phosphate (S1P). Sphingosine kinase can also phosphorylate sphinganine to produce sphinganine 1-phosphate (dihydrosphingosine 1-phosphate). S1P is either cleaved by S1P lyase (to hexadecenal and phosphoethanolamine) or converted back to ceramide, via sphingosine, by S1P phosphatase and CerS. In general terms, ceramide and sphingosine promote senescence or apoptotic cell death (1, 2), while S1P promotes cell growth (3).

Although initially regarded as simply intermediates in *de novo* ceramide biosynthesis, dihydroceramides are now recognised to have distinct biological functions, including in relation to autophagy, cell proliferation, hypoxia and several diseases (4). For example, dihydroceramides that accumulate in response to Degs1 inhibitors or the addition of exogenous dihydroceramides promote autophagy (5-7) and endoplasmic reticulum (ER) stress (7). However, some effects of Degs1 inhibitors on autophagy are independent of dihydroceramides (8). Nonetheless, genetic evidence supports a role for dihydroceramides in autophagy, which is linked with cell survival. Thus, Degs1 deficiency produces an anti-apoptotic effect via activation of AKT and autophagy (9). However, other studies have shown that inhibition of Degs1

promotes cell death, which may involve autophagy or apoptosis (7, 10). For example, apoptosis is induced by Degs1 inhibitors, including fenretinide and resveratrol (11-13) and this is prevented when enzymes upstream of Degs1 in the *de novo* ceramide synthesis pathway are inhibited (12). In contrast, inhibition of Degs1 or its genetic manipulation can produce resistance to apoptosis (7, 9, 14, 15).

One mechanism by which Degs1 might regulate cell survival is via endoplasmic reticulum (ER) stress, as lipid composition and particularly ceramide and dihydroceramide in the ER membrane activate ER stress responses (7, 16, 17). This can lead to an unfolded protein response (UPR), which is a survival process; however, a sustained UPR results in apoptosis (18). ER stress involves dissociation of the ER chaperone binding immunoglobulin protein (BiP) from the lumenal domains of ER stress sensors, which are protein kinase R-like ER kinase (PERK), inositol-requiring kinase 1 (IRE1α) and activating transcription factor 6 (ATF6). PERK is a kinase which phosphorylates eIF2α to inhibit protein synthesis. ER stress also initiates ER associated protein degradation (ERAD) to remove misfolded proteins. Recent studies have demonstrated that the SK2 inhibitors, ABC294640 or K145 induce ER stress with the latter increasing expression of XBP-1s and p-eIF2a (19). Targeting SK2 with K145 also contributed to ER stress and UPR activation induced by bortezomib, as evidenced by stimulation of the IRE1, JNK and p38 MAPK pathways, thereby resulting in potent synergistic apoptosis of myeloma cells *in vitro* (19). We have also shown that SKi induces an ER stress/UPR in T-cell lymphoblastic leukemic (T-ALL) cells that results in a protective cell survival autophagy (20). The effects of ABC294640 and SKi on Degs1 activity therefore raise the possibility for their involvement in both ‘survival’ promoting UPR or ‘apoptotic’ UPR.

Given the current controversy surrounding the role of Degs1 in regulating cell survival *versus* apoptosis, we have investigated the molecular basis for these opposing roles of Degs1. We demonstrate that Degs1 is subject to regulation by polyubiquitination induced by the sphingosine kinase inhibitor, SKi (2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole)) or fenretinide. Polyubiquitination of Degs1 promotes a 'gain of function', which enables activation of pro-survival pathways, including p38 MAPK, JNK and X-box protein-1s (XBP-1s). In contrast, another sphingosine kinase inhibitor, ABC294640 ((4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide)) failed to induce polyubiquitination of Degs1 at a concentration that increases *de novo* synthesis of ceramide and induces apoptosis via a Degs1-dependent mechanism. These findings are the first to suggest that the opposing functions of Degs1 on cell survival and apoptosis might be due to the polyubiquitination of Degs1.

Results

Effect of ABC294640 and SKi on the polyubiquitination of Degs1--The sphingosine kinase inhibitors, SKi and ABC294640 have previously been shown to reduce Degs1 activity, and this is associated with the proteasomal degradation of the enzyme in androgen-independent LNCaP-AI prostate cancer cells (21). We therefore, assessed the effect of these SK inhibitors on Degs1 expression levels in HEK293T cells. Native Degs1 is expressed as a 32 kDa protein in HEK293T cells, detected with an anti-Degs1 antibody on western blots (Fig. 1A). Treatment of HEK293T cells with SKi (10 μM, 24 h) induced the appearance of a ladder of higher molecular mass protein bands that immunoreacted with anti-Degs1 antibody (Fig. 1A) suggesting that SKi can stimulate the post-translational modification of Degs1. Confirmation of their identity was established using siRNA to knockdown Degs1 expression, which reduced the immunoreactive intensity of the 32 kDa protein and that of the laddered protein bands (Fig. 1B), e.g. 80% reduction in the expression of the 46 kDa post-translationally-modified form of Degs1 (Fig. 1B).

In contrast with SKi, treatment of HEK293T cells with ABC294640 (25 μM, 24 h) did not induce formation of the Degs1 ladder (Fig. 1A). Identical results were obtained with the parental HEK293 cell line (data not shown).

We also sought a second independent method to confirm the identity of the proteins in the ladder. In this regard, the post-translationally modified forms of Degs1 are present in a high-speed pellet fraction containing cell membranes (Fig. 1C) and can be released into a high-speed supernatant fraction using deoxycholate containing buffer but not NP-40 (data not shown). Using this method, the post-translationally modified forms of Degs1 were immunoprecipitated by the anti-Degs1 antibody (Fig. 1D).

Ubiquitination, which is catalysed by E3 ligase, will typically promote post-translational modification of proteins that can be resolved on SDS-PAGE as a ladder similar to that observed here for Degs1. We therefore, transfected HEK293T cells with a plasmid construct encoding HA-tagged ubiquitin and then treated these cells with SKi (10 μM, 24 h), after which Degs1 was immunoprecipitated with anti-Degs1 antibody and then probed with anti-HA antibody on western blots. SKi promoted the formation of a ladder of HA-ubiquitinated Degs1 forms (Fig. 1E). The HA-ubiquitinated Degs1 laddering in immunoprecipitates was similar to that detected in immunoprecipitates probed with anti-Degs1 antibody on western blots (Fig. 1D). Unpublished research in our laboratory has demonstrated that SKi promotes the ubiquitination of p53 which is catalysed Mdm2 (22). We therefore considered whether Mdm2 might be involved in regulating the ubiquitination of Degs1. Indeed, the formation of the Degs1 ladder in response to SKi was reduced when cells were pre-treated with a specific inhibitor of Mdm2, nutlin (Fig. 1F).

Effect of the proteasome inhibitor, MG132, on the polyubiquitination of Degs1--Given that the formation of the Degs1 ladder appears to reflect ongoing ubiquitin-proteasomal degradation, we assessed whether the proteasome inhibitor, MG132 induces the formation of the Degs1 ladder and, indeed, this was the case (Fig. 1A, Fig 2A). Interestingly, there were additional anti-Degs1 immuno-reactive protein bands with molecular mass below 32 kDa that are not formed in response to SKi (Fig. 1A, Fig. 2A). These smaller Degs1 forms might represent proteasomal degradation products that have accumulated due to inhibition of the proteasome by MG132. The identity of the proteins formed in response to MG132 was confirmed using Degs1 siRNA (Fig. 2A). These findings suggest that Degs1 is subject to dynamic turnover in HEK293T cells.

We therefore assessed whether combined treatment of cells with SKi and MG132 had any effect on the formation of the Degs1 ladder. In this regard, combined treatment increased the formation of Degs1 forms with a Mr > 50 kDa (Fig. 2A), which represent polyubiquitinated forms of Degs1. Formation of the Degs1 ladder is a feature of proteasome inhibition as a second proteasome inhibitor, bortezomib, which is chemically distinct from MG132, induced the same effect on Degs1 (Fig. 2B).

In order to establish whether Degs1 is subject to ubiquitin-proteasomal degradation in cancer cells, we treated PANC1 cancer cells with SKi and MG132. PANC1 cells express the native 32 kDa form of Degs1 (Fig. 2C) and SKi induced the appearance of higher molecular mass forms (53 kDa and 100-130 kDa, Fig. 2C). Treatment of cells with MG132 also promotes the formation of the Degs1 ladder that is similar to that observed in HEK293T cells (Fig. 2C). These findings indicate that, as in HEK293T cells, Degs1 is subject to a dynamic turnover via proteasomal degradation in PANC1 cells.

Therefore, we conclude that Degs1 is subject to polyubiquitination based on: (i) SKi induced formation of HA-ubiquitinated Degs1 forms that was detected in anti-Degs1 immunoprecipitates with anti-HA antibody on western blots, (ii) the formation of the Degs1 ladder is reduced by pre-treating cells with the Mdm2 inhibitor, nutlin; (iii) the proteasome inhibitors, MG132 and bortezomib induce formation of the Degs1 ladder, consistent with accumulation of polyubiquitinated forms of Degs1 that are trapped due to inhibition of the proteasome and (iv) the formation of the Degs1 ladder is reduced by siRNA knockdown of Degs1.

Assessment of the role of SK1 and SK2 in the polyubquitination of Degs1--We have previously shown that ABC294640 or SKi induce the proteasomal degradation of SK1 in cancer cells (21, 23). This was also the case in HEK293T cells where ABC294640 (25 μM) or SKi (10 μM) treatment reduced SK1 expression by ~ 80%, which was reversed by MG132 (Fig. 3A). The Degs1 ladder was not induced by siRNA knockdown of SK1 (Fig. 3B), which reduced SK1 protein expression by 70% (Fig. 3B). This suggests that SK1 is not involved in promoting formation of the Degs1 ladder in response to SKi. This is supported by the fact that ABC294640 (25 μM) induces proteasomal degradation of SK1 by 80% (Fig. 3A) from HEK293T cells, yet fails to promote the formation of the Degs1 ladder at this concentration. Further confirmation for the lack of a role for SK1 was obtained using the SK1 inhibitor PF-543 (24), which exhibits a K_i of 4 nM for SK1 inhibition. In this regard, PF-543 failed to induce formation of the Degs1 ladder (Fig. 3C).

Both ABC294640 and SKi also inhibit SK2 activity (25). Therefore, it is unlikely that SK2 inhibition is involved in promoting formation of the Degs1 ladder because ABC294640 (25 μM) failed to induce the Degs1 ladder (Fig. 1A). Moreover, siRNA knockdown of SK2 failed to induce formation of the Degs1 ladder (Fig. 3D). The siRNA knockdown of SK2 mRNA

transcript was 50% (Fig. 3D). In addition, neither SK1 nor SK2 knockdown affected formation of the Degs1 ladder in response to SKi (Fig. 3B, D).

Effect of SKi and ABC294640 on PARP cleavage and DNA synthesis--We next tested the effect of SKi and ABC294640 on growth and apoptosis of HEK293T cells. For this purpose, we measured cleavage of PARP and induction of CCAAT-enhancer-binding protein homologous protein (CHOP) expression as surrogate reporters for apoptosis (26), and [^{3}H]-thymidine incorporation into DNA to measure growth (although this does not exclude DNA repair). Treatment of HEK293T cells with ABC294640 (25 μM, 24 h) but not SKi (10 μM, 24 h) induced PARP cleavage, CHOP expression and reduced DNA synthesis (Fig. 4A-C), indicating that ABC294640 promotes apoptosis. The effect of ABC294640 on PARP cleavage and DNA synthesis was reversed by siRNA knockdown of Degs1 expression (Fig. 4A, C) indicating that Degs1 is involved in regulating apoptosis and DNA synthesis in response to ABC294640. We also noted that a higher concentration of ABC294640 (75 μM, 24 h) did produce a very weak Degs1 ladder (Fig. 4D), indicating that ABC294640 is less potent in inducing post-translational modification of Degs1 compared with SKi (Fig. 4A, C). We can therefore formally exclude the polyubiquitinated forms of Degs1 from inducing apoptosis because SKi fails to promote this response.

Given the effects of ABC294640 on SK1 expression (Fig. 3A) and SK2 activity (25), it was necessary to evaluate the role of these lipid kinases in regulating apoptosis in these cells. In this regard, SK1 knockdown with siRNA had no effect alone or on ABC294640-induced PARP cleavage (Fig. 4E), while SK2 knockdown (~50%) reduced cleavage of PARP in response to ABC294640 treatment (Fig. 4F). These findings suggest that SK2 and native Degs1 might collaborate to induce apoptosis in HEK293T cells in response to ABC294640.

Role of polyubiquitinated forms of Degs1 in regulating p38 MAPK and JNK activation--To further explore whether the polyubiquitinated form of Degs1 are protective against apoptosis, we assessed whether SKi might activate pro-survival signaling pathways. In this regard, SKi induced an increase in the phosphorylation of p38 MAPK and JNK in HEK293T cells (Fig. 5A). These are known as 'alarm signals' that might function to counter sustained UPR-induced cell death in this case. Indeed, treatment of cells with the JNK inhibitor, SP600125 induced the formation of cleaved PARP (Fig. 5B), while the p38 MAPK inhibitor, SB203580, reduced DNA synthesis (Fig. 5C), suggesting that JNK is protective against apoptosis while p38 MAPK promotes DNA synthesis. In addition, while SKi alone had no effect on DNA synthesis, it enhanced the inhibitory effect of SB203580 (Fig. 5C). This might suggest that SKi is also capable of activating growth inhibitory pathways but that these are suppressed when phosphorylated p38 MAPK levels are increased by SKi. Furthermore, treatment of HEK293T cells with Degs1 siRNA abolished the effect of SKi on the p38 MAPK and JNK pathways (Fig. 5A). In order for p38 MAPK/JNK signaling in response to SKi to be reduced by the siRNA knockdown of Degs1, the polyubiquitinated forms of Degs1 are likely to have acquired a 'gain of function'.

Degs1 siRNA reduces expression of both native and polyubiquitinated forms of Degs1 that accumulate in response to SKi (Fig. 2A). Therefore, the question arises as to which form(s) of Degs1 (native or the polyubquitinated forms) account for the increase in phosphorylated p38 MAPK and JNK levels. We can exclude the native Degs1 because the levels of phosphorylated p38 MAPK and JNK are not significantly reduced in cells treated with Degs1 siRNA alone (Fig. 5A). To evaluate the role of SK1 or SK2 in regulating p38 MAPK and JNK signaling, we used SK1 siRNA or SK2 siRNA. However, neither recapitulated the effect of SKi on p38 MAPK and JNK phosphorylation levels (Fig. 5D, E).

For completeness, we evaluated the effect of SB203580 (p38 MAPK inhibitor) and SP600125 (JNK inhibitor) on the polyubiquitination of Degs1 in response to SKi. Surprisingly, pre-treatment of cells with SB203580 (Fig. 5F) but not SP600125 (data not shown) decreased formation of the Degs1 ladder in response to SKi. Thus, p38 MAPK might be involved in a positive feedback loop that enhances the polyubiquitination of Degs1.

Effect of NAC, fenretinide and GT11 on the polyubiquitination of Degs1--Previous studies have proposed that SKi indirectly inhibits Degs1 activity via a mechanism involving an oxidative stress response and cytochrome B5 reductase (27). Similarly, fenretinde has been shown to inhibit Degs1 activity and induce oxidative stress (5, 28, 29). Therefore, we assessed whether the anti-oxidant, N-acetyl cysteine (NAC) and fenretinide can affect the post-translational modification of Degs1. In this regard, the pre-treatment of HEK293T cells with NAC reduced the formation of the Degs1 ladder in response to SKi (Fig. 6A). Fenretinide also induced the formation of post-translationally modified forms of Degs1 and pre-treatment with the anti-oxidant, NAC, reduced their formation (Fig. 6B). To more directly establish a link between compounds that can modulate Degs1 activity and formation of the Degs1 ladder, we used the competitive Degs1 inhibitor, GT11 ((N-[(1R,2S)-2-hydroxy-1-hydroxymethyl-2-(2-tridecyl-1-cyclopropenyl)ethyl]octanamide). GT11 has been shown to competitively inhibit Degs1 with a K_i = 6 μM (30) and treatment of primary neurones for 24 hours maximally inhibited the desaturation of ceramide at 100 nM (31). Higher concentrations of GT11 (10 μM) inhibit S1P lyase activity (31). Therefore, we treated HEK293T cells with GT11 (10 nM-10 μM), which failed to induce the Degs1 ladder (Fig. 6C). Taken together, these findings suggest that it might be necessary for SKi and fenretinide to induce oxidative stress in order to indirectly stimulate the polyubiquitination of Degs1 and that the effects are not likely a consequence of direct binding to Degs1.

The effect of ABC294640 and SKi on PERK and XBP-1s--Changes in lipid composition of the ER membrane, and particularly ceramide/dihydroceramide, is involved in ER stress. In addition, ER stress can contribute both pro-survival and apoptotic signals to the cell. Therefore, we considered whether the regulation of Degs1 by SKi and ABC294640 might involve this pathway. To evaluate this, we measured the levels of two ER stress effectors, namely, protein kinase R-like ER kinase (PERK) and X-box binding protein 1s (XBP-1s). Treatment of HEK293T cells with SKi induced a mobility shift in PERK on SDS-PAGE (Fig. 7A), which has previously been reported to be a consequence of phosphorylation (32). Tunicamycin (5 μg/ml) a *bona fide* inducer of ER stress also promoted a mobility shift in PERK (Fig. 7A).

The mobility shift in PERK induced by SKi was not reversed by siRNA knockdown of Degs1 (Fig. 7B). Therefore, the SKi-induced phosphorylation of PERK does not likely involve Degs1. Alternatively, inhibition of SK1 or SK2 activity by SKi might affect PERK phosphorylation. However, neither SK1 nor SK2 knockdown with siRNA recapitulated or modified the effects of SKi on PERK (Fig. 7C, D), thereby also possibly excluding these kinases. This is consistent with the finding that ABC294640, which inhibits SK2 (25) and induces proteasomal degradation of SK1 in HEK293T cells (Fig. 3A) failed to induce a mobility shift in PERK (Fig. 7A).

We next turned our attention to XBP-1s (Fig. 8). In this regard, neither ABC294640 nor SKi alone had any effect on XBP-1s expression. In contrast, MG132 induced a modest increase in the expression of XBP-1s. However, this was markedly enhanced by SKi (Fig. 8); significant because the combination of SKi and MG132 strongly stimulated formation of the Degs1 ladder, and particularly the accumulation of post-translationally modified Degs1 forms with a Mr > 50 kDa (Fig. 2A). Moreover, the siRNA knockdown of Degs1 reduced the MG132/SKi-induced

increase in XBP-1s expression (Fig. 8). In contrast, ABC294640 (25 μM) reduced the MG132-induced increase in XBP-1s levels (Fig. 8).

Effect of SKi and ABC294640 on sphingolipid levels--Since ABC294640 (25 μM) and SKi (10 μM) have a differential effect on Degs1, we investigated whether this is reflected in changes in sphingolipids. First, we looked at dihydroceramide levels. In this regard, both compounds only induced modest increases in dihydroceramide levels. ABC294640 (25 μM) induced an increase in C16:0, C18:1, C22:0, C24:1, C24:0 and C26:1 dihydroceramide levels, with C20:0 dihydroceramide levels approaching significance (Fig. 9A). SKi also induced an increase in dihydroceramide levels although, in contrast with ABC294640, only C16:0 and C24:0 dihydroceramides were elevated, while the increase in C24:1 dihydroceramide approached statistical significance (Fig. 9A). Hexosyl dihydroceramide levels were too low to produce a clear indication as to whether they were changed (data not shown).

Both SKi and ABC294640 are SK1/SK2 inhibitors and so we established their effect on long chain sphingoid bases. Treatment of the cells with SKi induced a reduction in sphinganine 1-phosphate levels with a decrease in S1P approaching statistical significance ($p=0.06$) (Fig. 9B), consistent with it inhibitory effect on SK1 and SK2 activity. This was accompanied by no effect on sphinganine levels, but a reduction in sphingosine levels. In contrast, ABC294640 induced an increase in sphingosine, sphinganine and S1P levels (Fig. 9B), which might reflect increased *de novo* synthesis and/or uptake, while reducing sphinganine 1-phosphate levels (Fig. 9B); the latter effect is consistent with SK inhibition. In line with the elevation of sphingosine and sphinganine levels, ABC294640 induced an increase in ceramide levels (Fig. 9C) and this might also reflect an induction of *de novo* biosynthesis and/or uptake from the medium. In this regard, ABC294640 induced an increase in various ceramide molecular species (including C14:0, C16:0,

C18:0, C18:1, C20:0, C22:0, C24:1, C24:0, C26:0 and C26:1) levels (Fig. 9C) and which is consistent with the ability of this compound to induce apoptosis. In contrast, SKi induced a decrease in C18:0 and C20:0 ceramides, while increasing C24:1 and C14:0 ceramides (Fig. 9C). SKi had little effect on total ceramides, suggesting that it probably fails to raise 'apoptotic' ceramides, thereby accounting for its lack of effect on PARP and CHOP (Fig. 4A, B). In terms of sphingomyelin levels, ABC294640 induced an increase in C20:0, C22:0, 24:0, 24:1 and C26:1 species with C18:0 approaching statistical significance, while SKi had no effect (Fig. 10A). ABC294640 also increased the levels of dihydrosphingomyelin (including C18:0, C22:0, C24:0, C26:1 with C24:1 approaching statistical significance ($p=0.054$)), while SKi increased the levels of C16:0 dihydrosphingomyelin (Fig. 10B).

Discussion

The major finding of this study is that Degs1 is regulated by polyubiquitination . Moreover, we demonstrate here that the native and polyubiquitinated forms of Degs1 exert opposing functions on cellular fate. Thus, the polyubiquitinated forms of Degs1 are linked with activation of pro-survival and growth signaling pathways e.g. JNK, XBP-1s and p38 MAPK (Fig. 11). The polyubquitinated forms of Degs1 appear to be in transit to proteasomal degradation, as accumulation of the forms is increased by combined treatment of the cells with the proteasome inhibitor, MG132 and SKi. Therefore, it is possible that some compounds remove Degs1 from cells via the ubiquitin-proteasomal degradation route and this might account for cell death induced by these compounds and which has been reported in the literature (7, 10-13). However, the polyubiquitinated forms themselves are endowed with a pro-survival function, and therefore, if the degradation rate is slow, then these forms might predominate and thereby account for the cell survival function of Degs1 (5-7, 9, 14, 15).

We have also demonstrated a role for oxidative stress, p38 MAPK (functioning in a positive feedback mechanism of regulation) and Mdm2 in promoting the polyubiquitination of Degs1. The mechanism by which p38 MAPK regulates the polyubiquitination of Degs1 remains to be determined. However, previous studies have shown that TNFα uses the p38 MAPK pathway to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle (33). There is additional evidence for the link between p38 MAPK and Mdm2. Thus, hypoxia has been shown to induce down-regulation of Mdm2 and this can be reduced by p38 MAPK inhibitors and by a dominant-interfering mutant of the p38-activating kinase mitogen-activated protein kinase kinase 3 (34).

In contrast with the polyubquitinated forms of Degs1, the native form of Degs1 appears to be linked with the induction of apoptosis (Fig. 11). In this regard, ABC294640 (25 μM) fails to induce the polyubiquitination of Degs1, but promotes apoptosis. A higher concentration of ABC294640 (75 μM) does stimulate the formation of the Degs1 ladder, suggesting that it is significantly less potent compared with SKi.

We have also established the effects of SKi and ABC294640 on sphingolipid levels that provides information concerning the effect of modulating Degs1, SK1 and SK2 activity on the survival of HEK293T cells. Treatment of cells with SKi increased C16:0 and C24:0 dihydroceramide levels. The increase in these species is relatively modest and can be accounted for by loss of some Degs1 via proteasomal degradation. However, the selective effect on these particular dihydroceramide species is somewhat surprising because this enzyme can catalyse the desaturation of many different molecular species of dihydroceramide. The dihydroceramide profile might, in fact, be an average of some loss of Degs1 via proteasomal degradation and some ‘gain of function’ acquired by the polyubiquitinated forms. Second, C14:0 and C24:1

ceramides were shown to be elevated in response to treatment of cells with SKi, and it is tempting to speculate that these might be formed from dihydroceramide by the polyubiquitinated Degs1 forms. In this regard, these molecular species might represent 'pro-survival' ceramides linked to activation of p38 MAPK/JNK and XBP-1s pathways. The 'gain of function' acquired by the polyubquitinated forms of Degs1 might require redistribution to a specific functional compartment with effectors of the survival pathways, where local regulation of discrete pools of specific dihydroceramide molecular species can take place. Indeed, Beauchamp *et al*. (35) have reported that Degs1 is N-myristoylated and targeted to the mitochondria, suggesting that there are two pools of Degs1, one in the ER and one in the mitochondria. This requires further investigation, as immunofluorescence analysis with anti-Degs1 antibody did not reveal any obvious change in the distribution of Degs1 with SKi (data not shown), although the polyubiquitinated forms of Degs1 might be resident in the ER because of the link with activation of the ER stress responsive effector, JNK. The modest effect of SKi on dihydroceramide levels might also suggest that the rate of proteasomal degradation of Degs1 and therefore, inactivation is rather slow in HEK293T cells, thereby allowing the intermediate polyubiquitinated forms to accumulate and to therefore instigate a persistent cell survival programme.

The treatment of HEK293T cells with ABC294640 increased both dihydroceramide and ceramide levels. This suggests an overall increase in flux through the *de novo* ceramide pathway or increased cellular uptake. The increased levels of dihydroceramide might effectively endow native Degs1 with a 'gain of function' by a substrate induction mechanism to promote formation of ceramide species that promote apoptosis. The changes in dihydroceramides with SKi and ABC294640 are consistent with previous studies which have reported that these compounds promote senescence of androgen-independent LNCaP-AI prostate cancer cells associated with the proteasomal degradation of SK1 and Degs1 (21) and increased p53 and p21 expression (21).

The inhibitory effect of SKi and ABC294640 on Degs1 activity results in increased dihydroceramide levels in prostate cancer cells (23, 36). SKi is proposed to also indirectly inhibit Degs1 via a cytochrome B5 reductase-dependent mechanism (27) and also increased dihydroceramide levels in ovarian cancer cells (37).

The treatment of cells with ABC294640 also increased both sphingosine and S1P, which is rather surprising given its effect in inducing proteasomal degradation of SK1 in HEK293T cells and characterisation as an SK2 inhibitor. Nevertheless, ABC29460 is a rather a weak inhibitor of SK2 (IC_{50} = 50 μM) (38). Therefore, there might be sufficient SK2 activity remaining in the presence of ABC294640 to drive S1P formation as a consequence of elevated sphingosine levels that accumulates in response to ABC294640. ABC94640 also reduced sphinganine 1-phosphate levels, which is consistent with its ability to induce the proteasomal degradation of SK1 in HEK293T cells. Therefore, these findings suggest that SK1 might be more important than SK2 in catalysing the formation of sphinganine 1-phosphate in these cells. Indeed, a similar reduction in sphinganine 1-phosphate levels is observed with SKi, which also induces the proteasomal degradation of SK1. SKi also reduces S1P levels, albeit moderately. A possible explanation for the difference in the action of ABC294640 and SKi on S1P levels then is that SKi is a much stronger inhibitor of SK2 activity (IC_{50} = 2 μM) (unpublished) compared with ABC294640, and therefore S1P levels might be predominantly regulated by SK2 in HEK293T cells.

The findings presented in this paper reveal that Degs1 is a very versatile enzyme and polyubiquitination might convert the enzyme from being pro-apoptotic to pro-survival. This might account for various conflicting reports on the role of Degs1 in promoting apoptosis or cell survival mediated by, for instance, autophagy (for review see (4)). There is evidence for similar regulation of CerS1 by the ubiquitin-proteasome degradation pathway. For instance, stress

induces translocation of CerS1 from the ER to Golgi and promotes its proteasomal degradation in HEK293 cells (39). Moreover, over-expression of CerS1 increases the activation of p38 MAPK by cisplatin in HEK293 cells (40). These findings are intriguing and suggest that CerS1 and Degs1 might operate through a similar linked ubiquitin-proteasomal degradation mechanism in response to chemical stress that results in activation of the p38 MAPK pathway.

The pro-survival function of Degs1 is likely to be dictated to by the lifetime of the polyubiquitinated forms of Degs1 in various different cellular systems. These findings might have important consequences when evaluating Degs1 as a target for therapeutic treatment of cancer. Our findings reveal possible modes of action by which drugs targeting Degs1 might act. First, drugs that prevent polyubiquitination of Degs1 might promote cell death if they also increase *de novo* biosynthesis of apoptotic ceramide. Second, drugs that accelerate the proteasomal degradation of the Degs1 might remove the pro-survival function of the polyubquitinated forms to potentially kill cancer cells via apoptotic/senescent pathways.

Materials and Methods

Materials--All general biochemicals were from Sigma (Poole, UK). ABC294640 (S7174) was from Stratech Scientific (UK). SKi (567731) was from Merck Biosciences (Nottingham, UK). MG132 (C2211), N-acetyl-L-cysteine (A7250), Nutlin (N6287) and Protein A-Sepharose® 4B, Fast Flow (P9424) were from Sigma (Poole, UK). SP600125 (BML-EI305) and SB203580 (BML-EI286) were from Enzo Life Sciences (UK). Bortezomib (sc-217785) and Fenretenide (HY-15373) were from Insight Biotechnology LTD (Insight Biotechnology LTD, UK). Anti-GAPDH (sc-47724) antibody was from Insight Biotechnology LTD (Insight Biotechnology LTD, UK); anti-Degs1 (ab185237) antibody was from Abcam; anti-P-SAPK/JNK (4671), anti-P-p38 MAPK (9211), anti-PARP (9542), anti-PERK (5683), anti-CHOP (5554) and anti-XBP-1s

(12782) antibodies were from New England Biolabs Ltd. (Hitchin, UK). Anti-SK1 antibody was custom made by Abcam. DharmaFECT^{TM} reagent, ON-TARGETplus SMARTpool® and Degs1, SK1 and SK2 siRNAs were from Dharmacon (Cromlington, UK). Scrambled siRNA (ALLSTARS Negative control) was from Qiagen (Crawley, UK). [Methyl-3H] thymidine (25Ci/mmol; 37MBq/ml) (NET027A) was from PerkinElmer (UK). The internal standard mix for quantitation of the sphingolipids by liquid chromatography, tandem mass spectrometry (LM-6002) was from Avanti Polar Lipids (Alabaster, AL, USA). GT11 was from Avanti Polar Lipids (Alabama, USA).

Cell Culture--HEK293T and PANC1 cells were maintained in DMEM/Glutamax supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) foetal bovine serum at 37°C with 5% CO_2 before treatment with compounds.

[^{3}H]-Thymidine incorporation--HEK293T cells (approx. 70% confluent) in 24 well plates were incubated with compounds or vehicle (DMSO, 0.1% v/v final), as detailed in the figure legends, for 20 hours prior to the addition of [^{3}H]-thymidine (9.25 kBq per well) for a further 5 hours. Incubations were terminated by removing the medium and immediately adding 1 ml of ice cold 10% (w/v) trichloroacetic acid and placed on ice for 10 minutes. This was replaced with a further 1 ml ice cold 10% (w/v) trichloroacetic acid for 10 minutes and repeated once more. Residual nuclear material was dissolved in 0.25 ml of 0.1% SDS/0.3 M NaOH. [^{3}H]-thymidine uptake was quantified by liquid-scintillation counting. Radiometric values (mean +/− SD) were obtained from 3 or more independent experiments.

siRNA Transfection--HEK293T cells were transiently transfected with siRNA constructs or scrambled siRNA (as a negative control) at a final concentration of 100 nM. Cells were

transfected at approximately 50-60% confluence and maintained in the transfection mixture for 48 hours.

Immunoprecipitation--Cells were placed in ice-cold lysis buffer (500 µl/3 wells) containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% (w/v) Na deoxycolate, 10% (v/v) Glycerol, 20 mM Tris-Base, 1 mg/ml BSA, 0.5 mM Na_3VO_4, 0.2 mM PMSF, leupeptin and aprotinin (both at 10 µg/ml); pH 8.0. The samples were homogenised using a 0.24 mm gauge needle and syringe and left to shake at 4°C for 60 minutes. After centrifugation to remove cell debris, 250 µl of supernatant was pre-cleared with 20 µl of protein A Sepharose beads (1:1 with lysis buffer; 20 minutes at 4°C) before subsequent immunoprecipitation by adding 2 µl of the anti-Degs1 antibody and 20 µl protein A Sepharose beads (1:1 with lysis buffer; 2 hours at 4°C). Immunoprecipitates were collected by centrifugation and washed three times with Buffer A (10 mM HEPES, 100 mM NaCl, 0.5% (v/v) NP-40 and 0.2 mM PMSF; pH 7.0) and three times with Buffer B (Buffer A without NP-40). The beads were then sedimented by centrifugation, the supernatant removed and 20 µl of Laemmli buffer [0.125 M Tris-HCl, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 4% (w/v) SDS and 0.004% (w/v) bromophenol blue; pH 6.7] added to the beads, which were then heated at 100°C for 3 minutes. The samples were then subjected to SDS-PAGE.

Western Blotting--Upon treatment, HEK293T cells were lysed in sample buffer containing 62.5 mM Tris-HCl (pH 6.7), 0.5 M sodium pyrophosphate, 1.25 mM EDTA, 1.25% (w/v) sodium dodecyl sulphate, 0.06% (w/v) bromophenol blue, 12.5% (v/v) glycerol and 50 mM dithiothreitol. Proteins were separated on a 10% (v/v) acrylamide/bisacrylamide gel, and transferred to nitrocellulose Hybond membrane (GE Healthcare). Membranes were blocked in 5% (w/v) BSA (Fisher) in TBST buffer containing 20 mM Tris-HCl (pH 7.5), 48 mM NaCl,

0.1% (v/v) Tween 20 for 1 hour at room temperature prior to incubation with primary antibody (diluted in blocking buffer) overnight at 4ºC. Following three washes in TBST, membranes were incubated with horse radish peroxidase conjugated anti-mouse or anti-rabbit IgG secondary antibody, as required, for 1 hour at room temperature. Immunoreactive protein bands were visualised using enhanced chemiluminescence.

RNA extraction--Total RNA from the cell samples were isolated using an RNeasy Plus Mini kit (Qiagen, Manchester, UK) as per the manufacturer's instructions. Spectrophotometric analysis by a Nanodrop 2000C (ThermoFisher Scientific, Renfrew, UK) obtained the RNA concentrations and all of the RNAs had $A^{260/280}$ ratios of approximately 2.1. The RNAs were stored at -80°C until required

Gene expression analysis by Quantitative Real-time PCR--RNA from each cell sample were reverse transcribed to complementary DNA (cDNA) with Tetro cDNA synthesis kit (Bioline, London, UK) along with aliquots processed without reverse transcriptase to function as no-template controls for real-time quantitative polymerase chain reaction (RT-qPCR) and were stored at -20°C. RT-qPCR was conducted using PrimeTime® qPCR Probe assays for *SPHK2* (Assay name: Hs.PT.58.3726704) and *GAPDH* (Assay name: Hs.PT.39a.22214836) as the reference gene with PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, Leuven, Belgium). Thermal cycling was carried out on an ABI StepOnePlus real-time PCR system (Applied Biosystems, Warrington, UK) under the following protocol: Polymerase activation at 95°C for 3 mins followed by 40 cycles of denaturation at 95°C for 5 sec and annealing/extension at 60°C for 30 sec. Relative quantification of gene expression analysis between the samples treated with the control scrambled siRNA and those treated with *SPHK2*

siRNA used the $2^{\wedge-\Delta\Delta Ct}$ method to determine the percentage knockdown of *SPHK2* expression (41).

Lipidomics--HEK293T cells were treated with vehicle (DMSO, 0.1% v/v final), ABC294640 (25 μM) or SKi (10 μM) for 24 hours, then carefully rinsed twice with 1 ml ice cold PBS before being scraped into ice cold PBS. Cells were pelleted by centrifugation (180 *g*, 4^{o}C, 3 minutes) and the supernatant carefully removed. The cell pellet was snap frozen in liquid nitrogen for 5 seconds before being stored in -80^{o}C for sphingolipid analysis, which was conducted as described previously (42, 43) using liquid chromatography, electrospray-ionization tandem mass spectrometry and multiple reaction monitoring for quantitation.

Densitometry--Quantification of immunoreactive bands was performed using ImageJ.

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Figures

Fig. 1 Effect of ABC294640 or SKi on Degs1 protein laddering in HEK293T cells. Cells maintained in serum and grown to 70% confluence were treated with ABC294640 (25 μM) or SKi (10 μM) for 24 h. In certain cases, cells were treated with scrambled or Degs1 siRNA (100 nM) for 48 h or nutlin (10 μM) or MG132 (10 μM) for 30 min prior to treatment with SKi or ABC294640. Where indicated, cells were transfected with HA-ubiquitin plasmid construct for 8hr prior to treatment with or without SKi. (A) Western blot probed with anti-Degs1 antibody showing the effect of SKi or ABC294640, in the presence or absence of MG132, on Degs1 laddering. (B) Western blot probed with anti-Degs1 antibody showing the effect of Degs1 siRNA on formation of the Degs1 ladder in response to SKi. The bar graph demonstrates the reduction in polyubiquitinated Degs1 (Mr=46 kDa) expression levels by

Degs1 siRNA in the presence of SKi. Results are expressed as Degs1:GAPDH ratio (mean +/- SEM for n=3 independent experiments; * $p<0.01$ *versus* scr siRNA/SKi) (C) Western blot probed with anti-Degs1 antibody showing the localisation of the Degs1 ladder in the pellet fraction. (D) Western blot probed with anti-Degs1 antibody showing the presence of the Degs1 ladder in anti-Degs1 immunoprecipitates. (E) Western blot showing the formation of HA-ubiquitinated forms of Degs1 in response to SKi, and detected in anti-Degs1 immunoprecipitates with anti-HA antibody. Also shown is a western blot of lysates from cells transfected with vector- or HA-ubiquitin plasmid construct probed with anti-HA antibody. (F) Western blot probed with anti-Degs1 antibody showing the effect of nutlin on formation of the Degs1 ladder in response to SKi. Blots A and B and F were reprobed for GAPDH using anti-GAPDH antibody to ensure comparable protein loading. Results are representative of at least 3 independent experiments.

Fig. 2 MG132- and bortezomib-induced formation of the Degs1 ladder. Cells maintained in serum and grown to 70% confluence were treated with ABC294640 (25 μM) or SKi (10 μM) for 24 h. In certain cases, cells were treated with scrambled siRNA or Degs1 siRNA (each at a final concentration of 100 nM) for 48 h or MG132 (10 μM) for 30 min prior to treatment with ABC294640 or SKi. Cells were also treated with Bortezomib (5 or 10 nM) for 24 h. (A) Western blot probed with anti-Degs1 antibody showing the effect of Degs1 siRNA on Degs1 laddering induced by MG132 in the presence or absence of SKi or ABC294640. (B) Western blot probed with anti-Degs1 antibody showing the effect of Bortezomib on Degs1 laddering. (C) Western blot showing the formation of the Degs1 ladder in response to SKi or MG132 in PANC1 cells. Blots were reprobed for GAPDH using anti-GAPDH antibody to ensure comparable protein loading. Results are representative of at least 3 independent experiments.

Fig. 3 Assessment of the role of SK1 and SK2 in formation of the Degs1 ladder. Cells maintained in serum and grown to 70% confluence were treated with ABC294640 (25 μM) or SKi (10 μM) or PF-543 (100 nM) for 24 h. In certain cases, cells were treated with SK1 or SK2 siRNA (each at a final concentration of 100 nM) for 48 h or MG132 (10 μM) for 30 min prior to treatment with SKi or ABC294640. (A) Western blot probed with anti-SK1 antibody showing the effect of ABC294640 or SKi on SK1 expression and reversal by MG132. The bar graph demonstrates the reduction in SK1 expression levels with SKi or ABC294640. Results are expressed as SK1:GAPDH ratio (mean +/- SEM for n=6 independent experiments; * $p<0.01$ *versus* control). (B) Western blot probed with anti-Degs1 antibody showing the lack of effect of SK1 siRNA on formation of the Degs1 ladder in response to SKi. Also shown is a western blot and bar graph demonstrating reduction in SK1 protein expression with SK1 siRNA. Results are expressed as SK1:GAPDH ratio (mean +/- SEM for n=5 independent experiments; * $p<0.01$ *versus* scr siRNA). (C) Western blot probed with anti-Degs1 antibody showing the lack of PF-543 on formation of the Degs1 ladder. (D) Western blot probed with anti-Degs1 antibody showing the lack of effect of SK2 siRNA on formation of the Degs1 ladder in response to SKi. Also shown is the RT-qPCR analysis demonstrating reduction in SK2 mRNA transcript with SK2 siRNA. Results are expressed as % decrease in expression of SK2 (mean +/- SEM for n=3 independent determinations; * $p<0.01$ *versus* scr siRNA). Blots were reprobed for GAPDH using anti-GAPDH antibody to ensure comparable protein loading and are representative of at least 3 independent experiments.

Fig. 4 Effect of ABC294640 or SKi on PARP cleavage in HEK293T cells. Cells maintained in serum and grown to 70% confluence were treated with ABC294640 (25-75

μM) or SKi (10 μM) for 24 h or times indicated. In certain cases, cells were treated with scrambled or Degs1 or SK1 or SK2 siRNA (each final concentration of 100 nM) for 48 h prior to treatment with ABC294640 or SKi. (A) Western blot probed with anti-PARP antibody showing the effect of Degs1 siRNA, SKi or ABC294640 (25 μM) on PARP cleavage. (B) Western blot probed with anti-CHOP antibody showing the effect of SKi ((10 μM) or ABC294640 (25 μM) or MG132 (10 μM) on CHOP expression. Also shown in the time dependent increase in CHOP expression in response to ABC294640. (C) Bar graph showing the effect of Degs1 siRNA, SKi or ABC294640 (25 μM) on DNA synthesis. * $p < 0.05$ for ABC294640/scrambled siRNA *versus* control/scrambled siRNA and ** $p < 0.05$ for ABC294640/scrambled siRNA *versus* ABC294640/Degs1 siRNA for n=3 independent experiments. (D) Western blot probed with anti-Degs1 antibody showing the effect of ABC294640 (25, 50 or 75 μM) on Degs1 laddering. (E) Western blot probed with anti-PARP antibody showing the effect of SK1 siRNA, SKi or ABC294640 (25 μM) on PARP cleavage. (F) Western blot probed with anti-PARP antibody showing the effect of SK2 siRNA, SKi or ABC294640 (25 μM) on PARP cleavage. Blots were reprobed for GAPDH using anti-GAPDH antibody to ensure comparable protein loading. Results (A, B, D, E, F) are representative of at least 3 independent experiments.

Fig. 5 p38 MAPK and JNK signaling. Cells maintained in serum and grown to 70% confluence were treated with SKi (10 μM) for 24 h. In certain cases, cells were treated with scrambled or Degs1 or SK1 or SK2 siRNA (each at a final concentration of 100 nM) for 48 h or SB203580 (10 μM) or SP600125 (20 μM) for 30 min prior to treatment with and without SKi. (A) Western blot probed with anti-phospho p38 MAPK or anti-phospho JNK antibodies showing the effect of Degs1 siRNA on the phosphorylation levels of p38 MAPK and JNK in response to SKi. (B) Western blot probed with anti-PARP antibody showing the effect of

SB203580 or SP600125 on PARP cleavage. (C) Bar graph showing the effect of SKi with or without SB203580 on DNA synthesis. * $p < 0.05$ for SB203580 *versus* control and ** $p < 0.05$ for SB203580/SKi *versus* SB203580 alone for n=3 independent experiments. (D, E) Western blot probed with anti-phospho p38 MAPK and anti-phospho JNK antibodies showing no effect of SK1 siRNA (D) or SK2 siRNA (E) on the phosphorylation levels of p38 MAPK and JNK. (F) Western blot probed with anti-Degs1 antibody showing the effect of SB203580 on Degs1 laddering in response to SKi. Blots were reprobed for GAPDH using anti-GAPDH antibody, anti-p38 MAPK or anti-JNK antibody to ensure comparable protein loading. Results (A, B, D, E, F) are representative of at least 3 independent experiments.

Fig. 6 Oxidative stress and polyubiquitination of Degs1. Cells maintained in serum and grown to 70% confluence were treated with SKi (10 μM) or MG132 (10 μM) or fenretinide (1 μM) or GT-11 (10 nM-10 μM) for 24 h. In certain cases, cells were treated with NAC (10 mM) for 30 min prior to treatment with SKi or fenretinide or GT11. (A, B) western blot probed with anti-Degs1 antibody showing the effect NAC on Degs1 laddering in response to SKi (A) or fenretinide (B). (C) Western blot showing the lack of effect of GT11 on Degs1 laddering. Blots were reprobed for GAPDH using anti-GAPDH antibody to ensure comparable protein loading. Results are representative of at least 3 independent experiments.

Fig. 7 PERK signaling. Cells maintained in serum and grown to 70% confluence were treated with ABC294640 (25 μM) or SKi (10 μM) for 24 h or tunicamycin (5 μg/ml) for up to 8 h. In certain cases, cells were treated with scrambled or Degs1 or SK1 or SK2 siRNA (each at a final concentration of 100 nM) for 48 h prior to treatment with SKi. Western blots probed with anti-PERK antibody showing the effect of (A) SKi or ABC294640 or tunicamycin, (B) Degs1 siRNA, (C) SK1 siRNA or (D) SK2 siRNA on the mobility shift of

PERK induced by SKi. Blots were reprobed for GAPDH or actin using anti-GAPDH or anti-actin antibodies to ensure comparable protein loading. Results are representative of at least 3 independent experiments.

Fig. 8 Regulation of XBP-1s expression. Cells maintained in serum and grown to 70 % confluence were treated with ABC294640 (25 μM) or SKi (10 μM) for 24 h. In certain cases, cells were treated with scrambled or Degs1 siRNA (final concentration of 100 nM) for 48 h or MG132 (10 μM) for 30 min prior to treatment with SKi or ABC294640. Western blot probed with anti-XBP-1s antibody showing the effect of Degs1 siRNA treatment on the induction of XBP-1s by MG132 in the presence and absence of SKi or ABC294640. Blots were reprobed for GAPDH using anti-GAPDH antibody to ensure comparable protein loading. Results are representative of at least 3 independent experiments.

Fig. 9 Effect of ABC294640 and SKi on dihydroceramide, sphingoid base and ceramide levels. Cells maintained in serum and grown to 70% confluence were treated with ABC294640 (25 μM) or SKi (10 μM) for 24 h before snap-freezing. Lipid extracts were analysed by LC-MS for different molecular species of (A) dihydroceramide; (B) sphingoid bases; (C) ceramides. * $p < 0.05$ for ABC294640 or SKi *versus* control for n=3 independent samples. Sphinganine and sphinganine 1-phosphate are denoted here as dhSph and dhS1P respectively. For (A) and (C) the x-axis annotates different N-acyl chain lengths and double bond molecular species.

Fig. 10 Effect of ABC294640 and SKi on sphingomyelin and dihydrosphingomyelin levels. Cells maintained in serum and grown to 70% confluence were treated with ABC294640 (25 μM) or SKi (10 μM) for 24 h before snap-freezing. Lipid extracts were

analysed by LC-MS for different molecular species of (A) sphingomyelin and (B) dihydrosphingomyelin. * $p < 0.05$ for ABC294630 or SKi *versus* control for n=3 independent samples. The x-axis annotates different N-acyl chain lengths and double bond molecular species.

Fig. 11 Schematic showing the different roles of native and polyubiquitinated Degs1 forms in regulating pro-survival/growth and apoptotic pathways in HEK293T cells. Native Degs1 is proposed to regulate formation of apoptotic ceramide species, while polyubiquitinated Degs1 forms are proposed to regulate formation of pro-survival/growth ceramide species.

Fig. 1

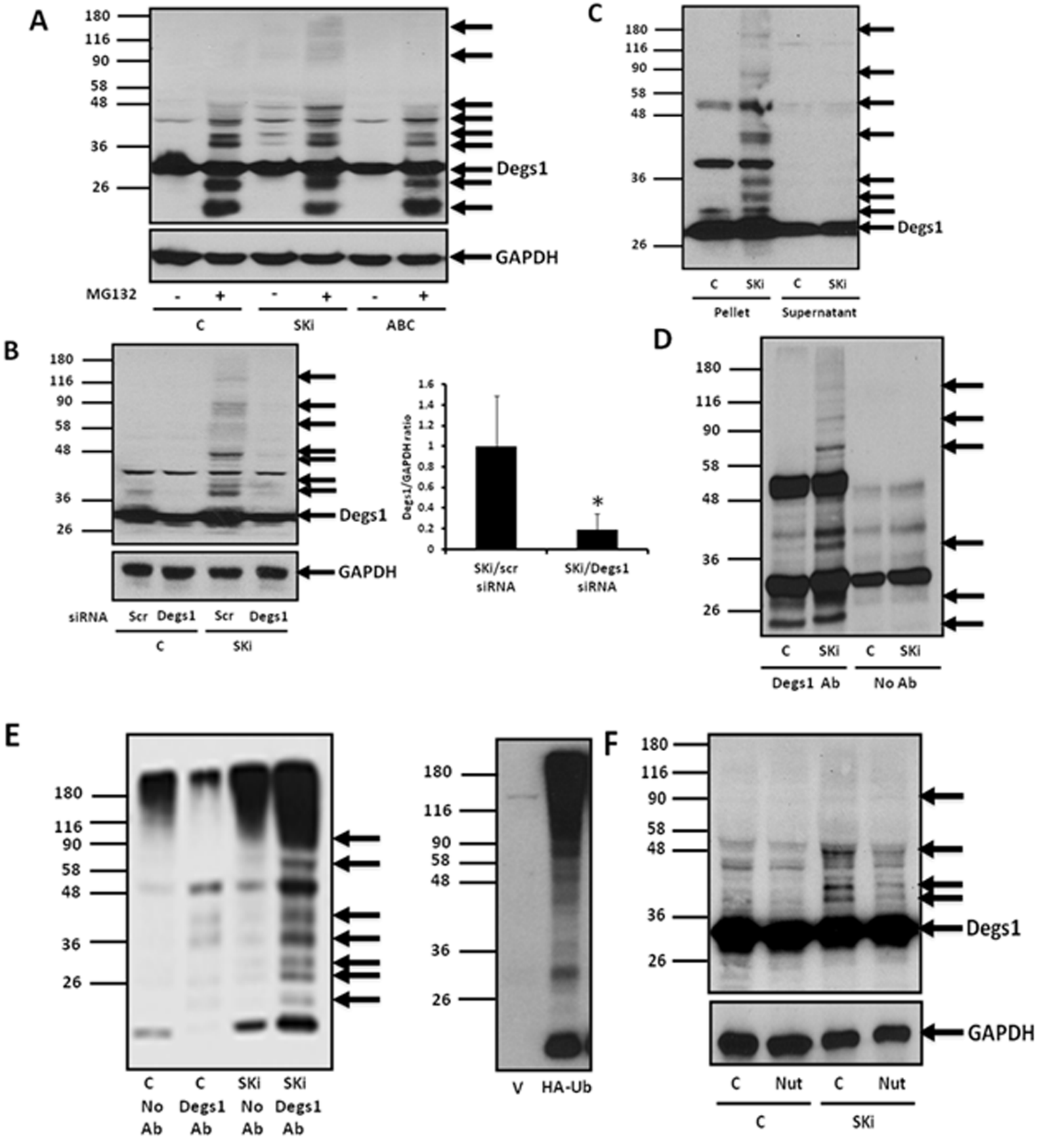

A
180
116
90
58
48
36
26
Degs1
GAPDH
MG132
- + - + - +
C
SKi
ABC
C
180
116
90
58
48
36
26
Degs1
C SKi C SKi
Pellet
Supernatant
B
180
116
90
58
48
36
26
Degs1
GAPDH
siRNA Scr Degs1 Scr Degs1
C
SKi
Degs1/GAPDH ratio
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
SKi/scr siRNA
SKi/Degs1 siRNA
D
180
116
90
58
48
36
26
C SKi C SKi
Degs1 Ab
No Ab
E
180
116
90
58
48
36
26
C No Ab
C Degs1 Ab
SKi No Ab
SKi Degs1 Ab
180
116
90
58
48
36
26
V
HA-Ub
F
180
116
90
58
48
36
26
Degs1
GAPDH
C Nut C Nut
C
SKi

Fig. 2

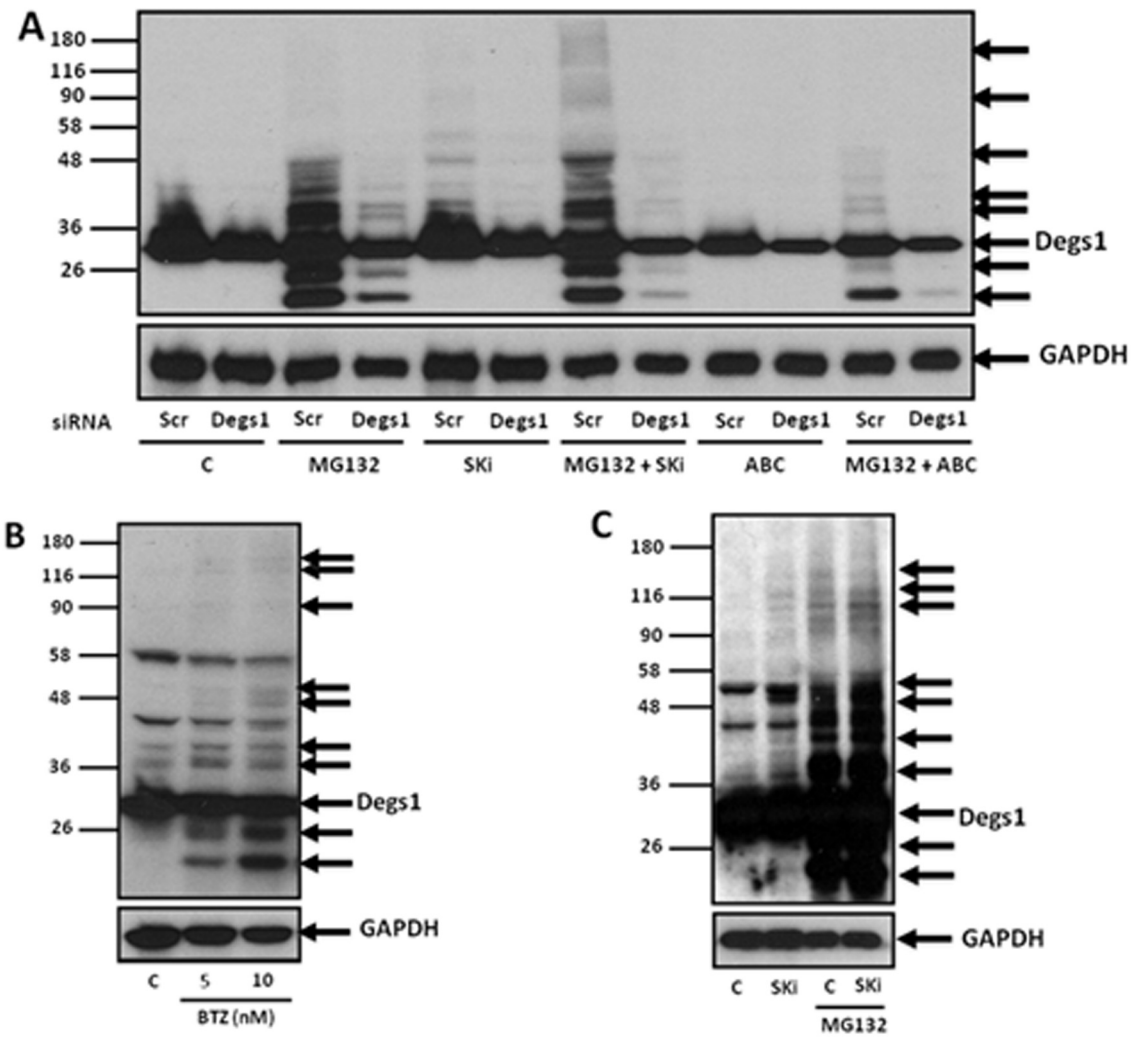

A
180
116
90
58
48
36
26
Degs1
GAPDH
siRNA
Scr
Degs1
C
MG132
SKi
MG132 + SKi
ABC
MG132 + ABC
B
180
116
90
58
48
36
26
Degs1
GAPDH
C
5
10
BTZ (nM)
C
180
116
90
58
48
36
26
Degs1
GAPDH
C
SKi
MG132

Fig. 3

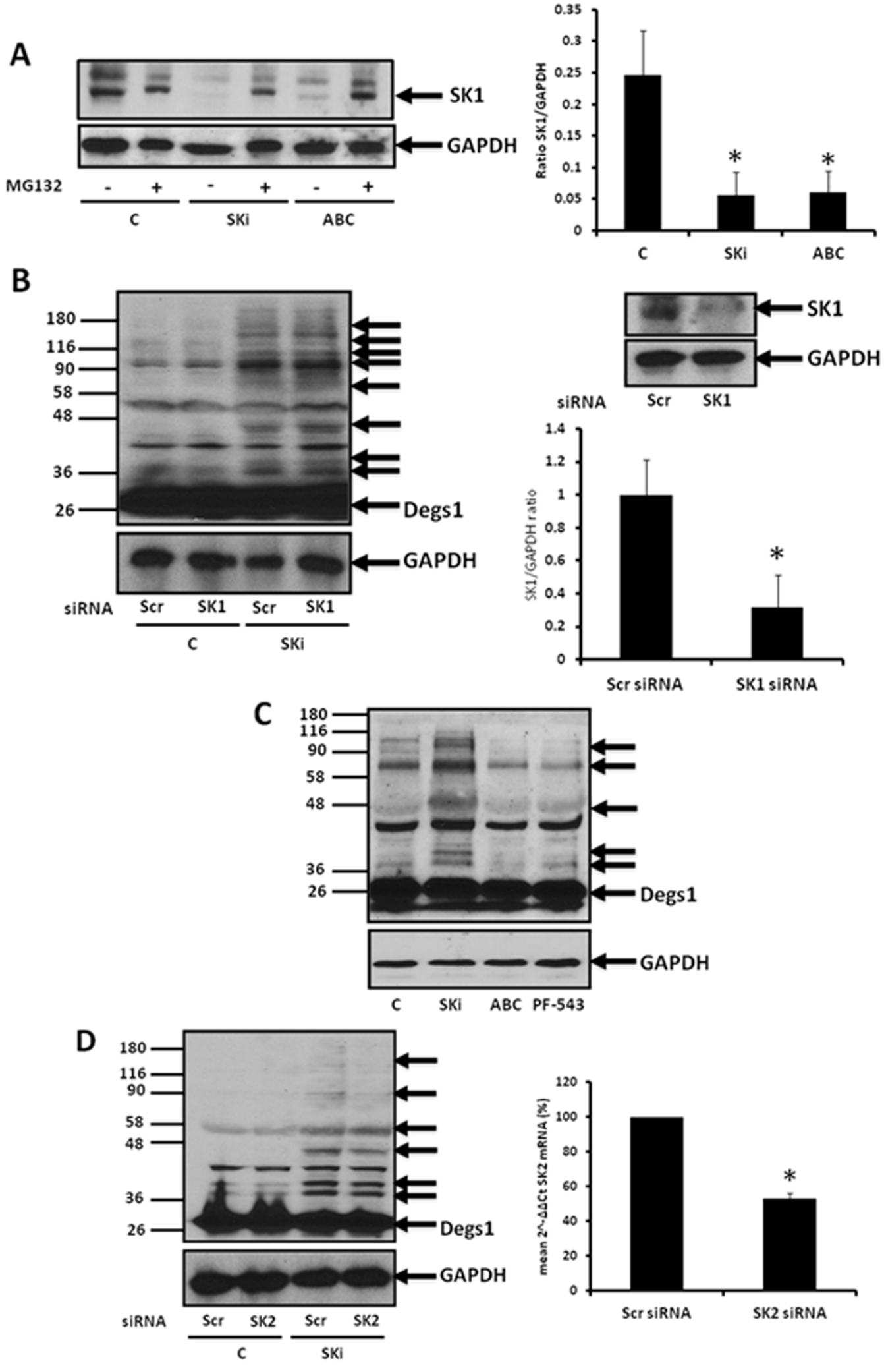

A
SK1
GAPDH
MG132
- + - + - +
C
SKi
ABC
Ratio SK1/GAPDH
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*
C
SKi
ABC
B
180
116
90
58
48
36
26
Degs1
GAPDH
siRNA
Scr SK1 Scr SK1
C
SKi
SK1
GAPDH
siRNA
Scr SK1
SK1/GAPDH ratio
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Scr siRNA
SK1 siRNA
C
180
116
90
58
48
36
26
Degs1
GAPDH
C SKi ABC PF-543
D
180
116
90
58
48
36
26
Degs1
GAPDH
siRNA
Scr SK2 Scr SK2
C
SKi
mean 2^-ΔΔCt SK2 mRNA (%)
120
100
80
60
40
20
0
*
Scr siRNA
SK2 siRNA

Fig. 4

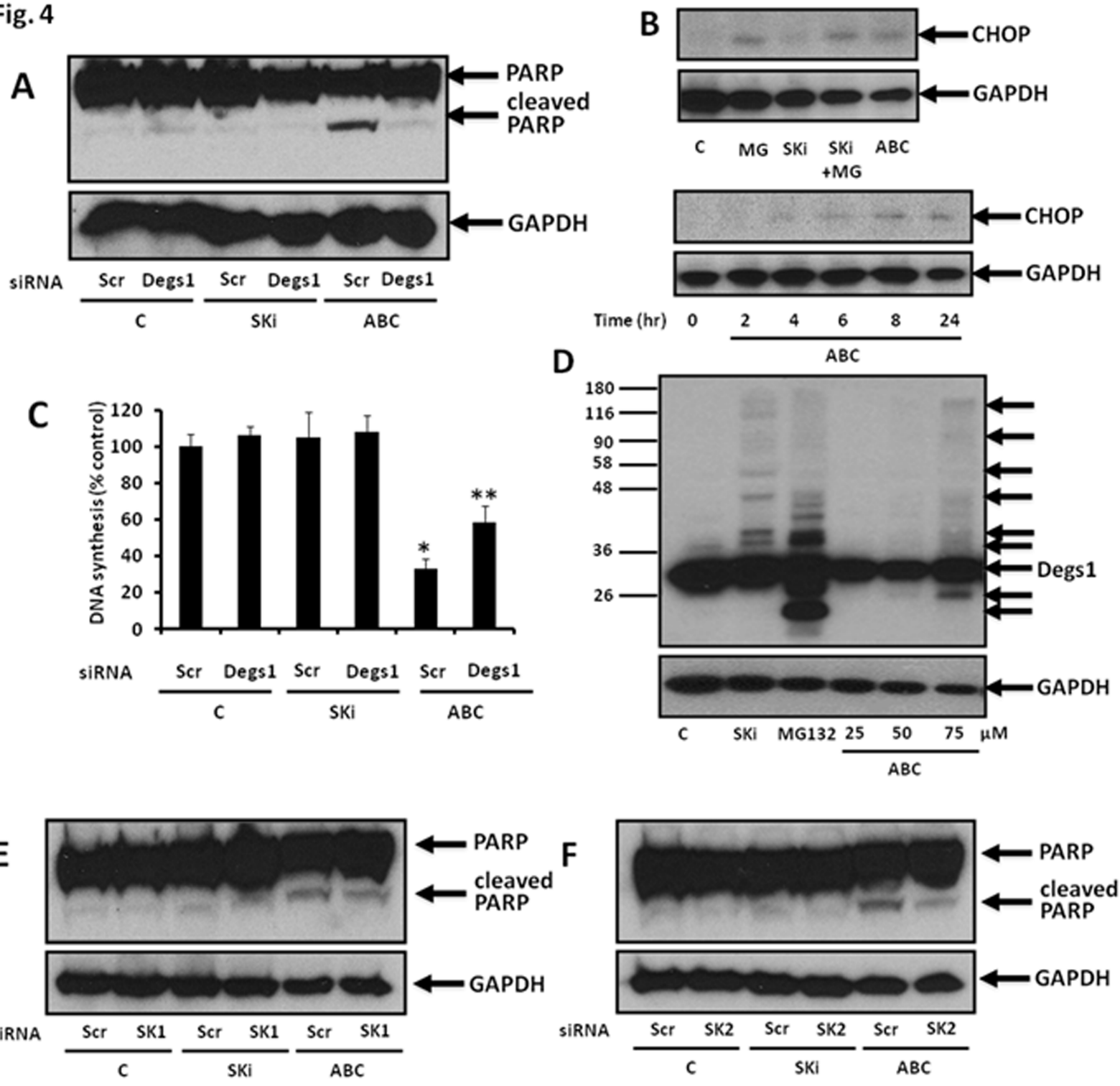

A
PARP
cleaved PARP
GAPDH
siRNA Scr Degs1 Scr Degs1 Scr Degs1
C SKi ABC
B
CHOP
GAPDH
C MG SKi SKi +MG ABC
CHOP
GAPDH
Time (hr) 0 2 4 6 8 24
ABC
C
DNA synthesis (% control)
120 100 80 60 40 20 0
*
**
siRNA Scr Degs1 Scr Degs1 Scr Degs1
C SKi ABC
D
180 116 90 58 48 36 26
Degs1
GAPDH
C SKi MG132 25 50 75 μM
ABC
E
PARP
cleaved PARP
GAPDH
siRNA Scr SK1 Scr SK1 Scr SK1
C SKi ABC
F
PARP
cleaved PARP
GAPDH
siRNA Scr SK2 Scr SK2 Scr SK2
C SKi ABC

Fig. 5

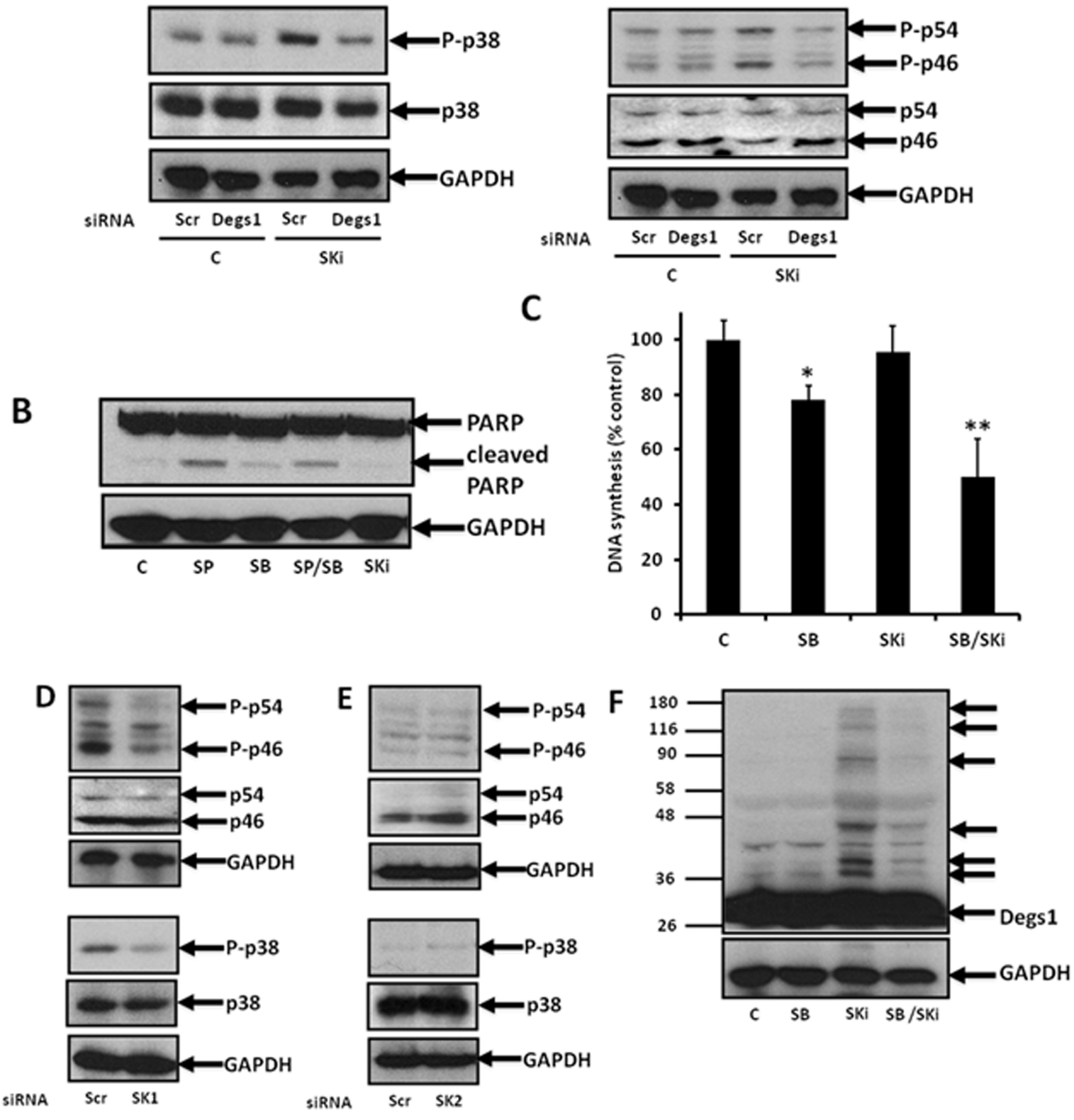

A
P-p38
p38
GAPDH
siRNA
Scr
Degs1
Scr
Degs1
C
SKi
P-p54
P-p46
p54
p46
GAPDH
siRNA
Scr
Degs1
Scr
Degs1
C
SKi
B
PARP
cleaved PARP
GAPDH
C
SP
SB
SP/SB
SKi
C
DNA synthesis (% control)
100
80
60
40
20
0
*
**
C
SB
SKi
SB/SKi
D
P-p54
P-p46
p54
p46
GAPDH
P-p38
p38
GAPDH
siRNA
Scr
SK1
E
P-p54
P-p46
p54
p46
GAPDH
P-p38
p38
GAPDH
siRNA
Scr
SK2
F
180
116
90
58
48
36
26
Degs1
GAPDH
C
SB
SKi
SB /SKi

Fig. 6

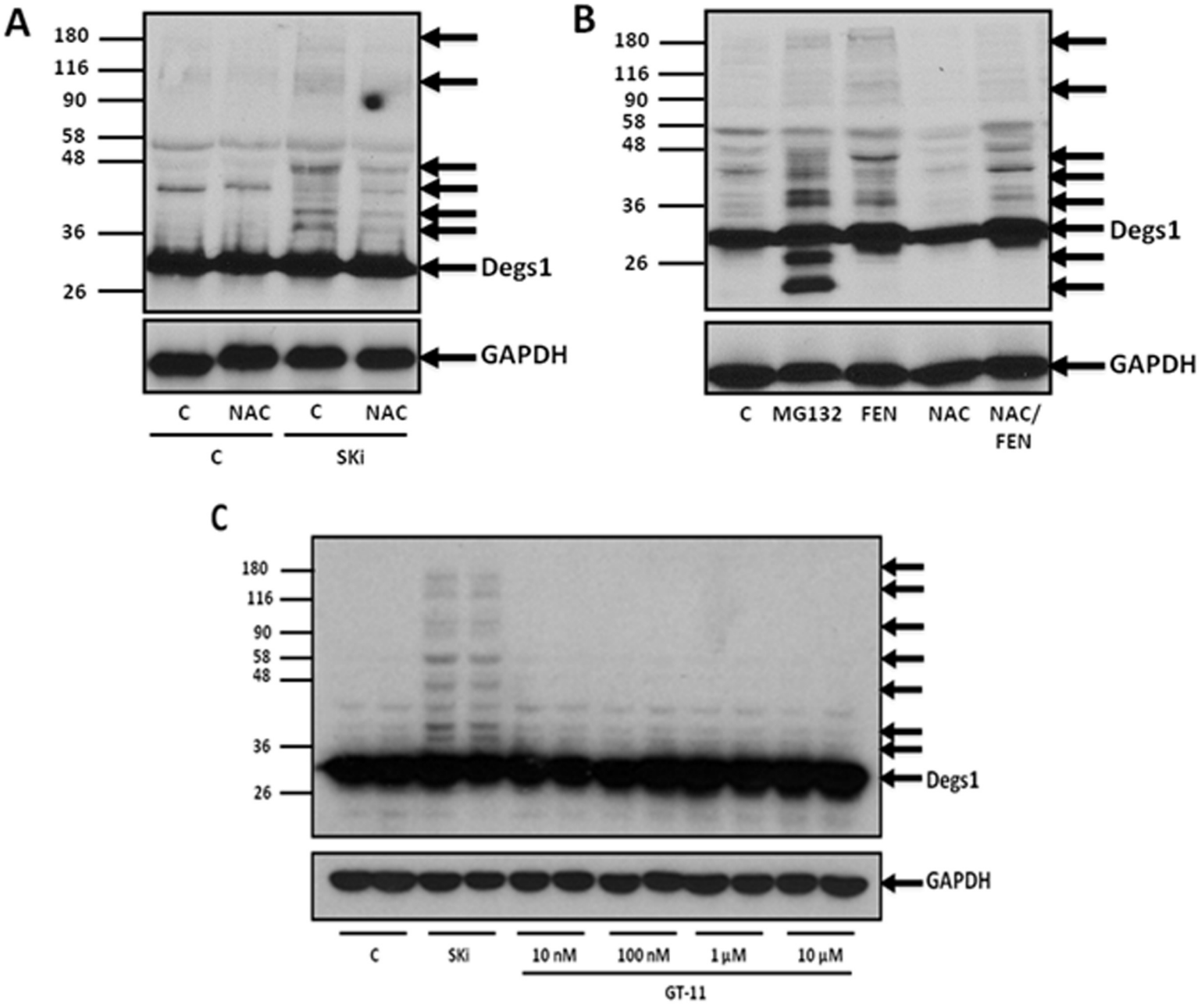

A
180
116
90
58
48
36
26
Degs1
GAPDH
C
NAC
C
NAC
C
SKi
B
180
116
90
58
48
36
26
Degs1
GAPDH
C
MG132
FEN
NAC
NAC/
FEN
C
180
116
90
58
48
36
26
Degs1
GAPDH
C
SKi
10 nM
100 nM
1 μM
10 μM
GT-11

Fig. 7

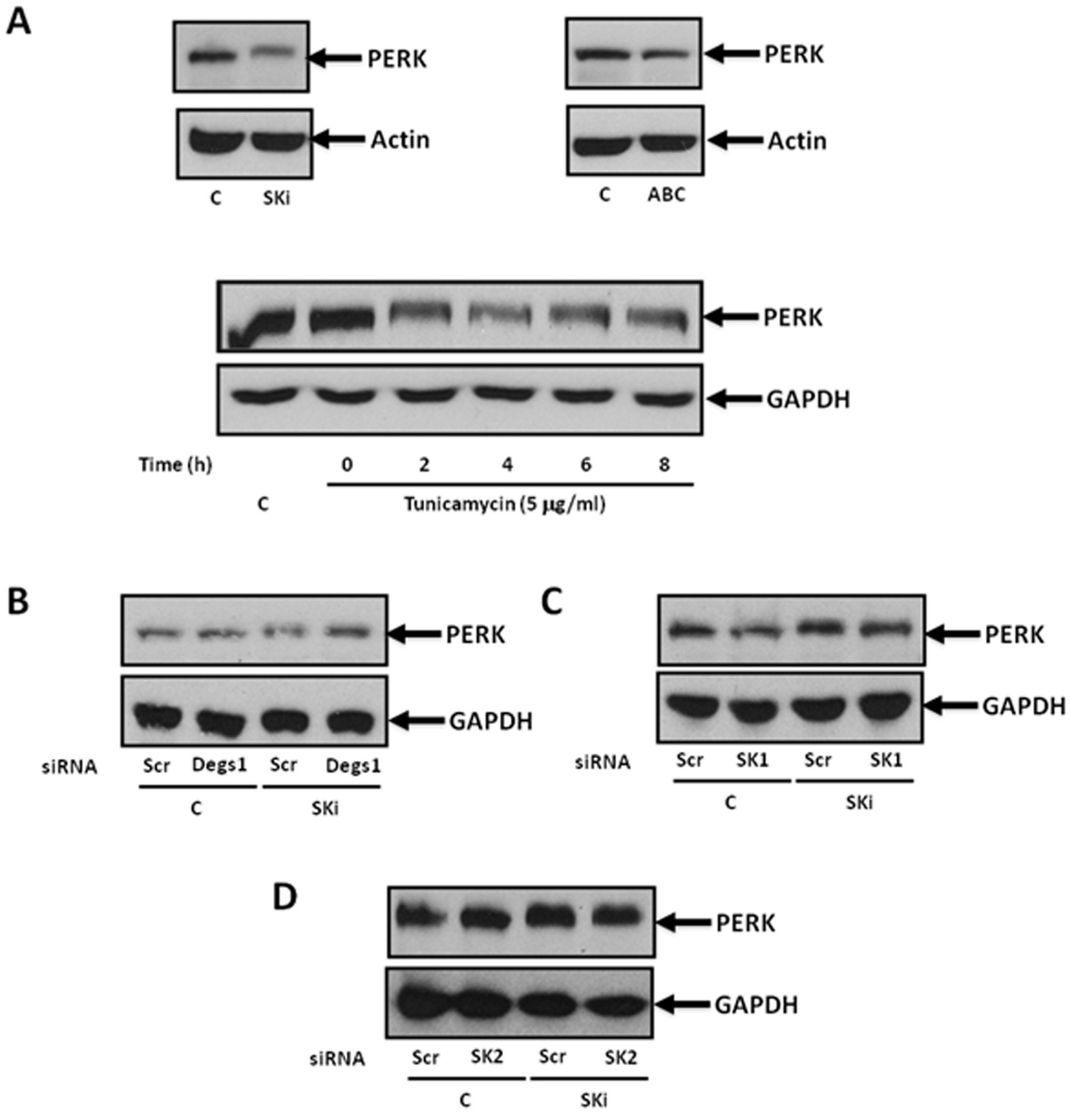

A
PERK
Actin
C
SKi
PERK
Actin
C
ABC
PERK
GAPDH
Time (h)
0
2
4
6
8
C
Tunicamycin (5 μg/ml)
B
PERK
GAPDH
siRNA
Scr
Degs1
Scr
Degs1
C
SKi
C
PERK
GAPDH
siRNA
Scr
SK1
Scr
SK1
C
SKi
D
PERK
GAPDH
siRNA
Scr
SK2
Scr
SK2
C
SKi

Fig. 8

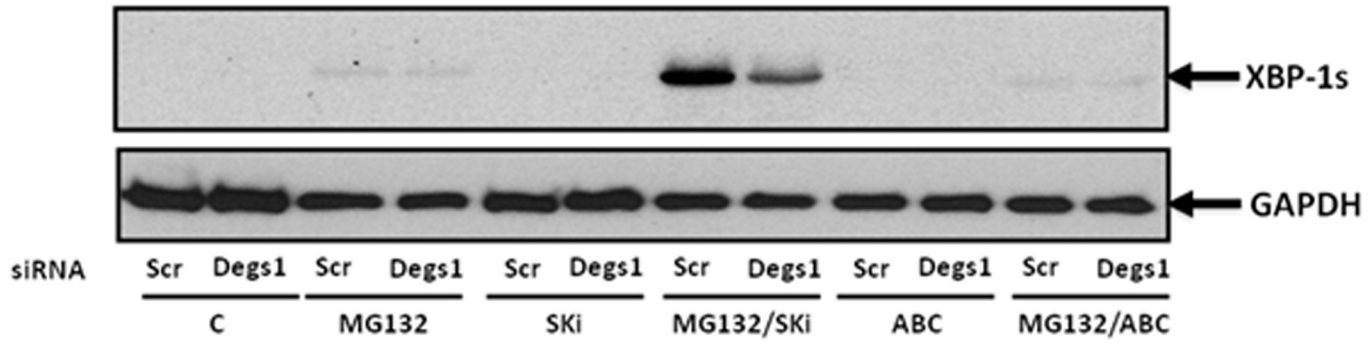

XBP-1s
GAPDH
siRNA
Scr
Degs1
C
MG132
SKi
MG132/SKi
ABC
MG132/ABC

Fig. 9

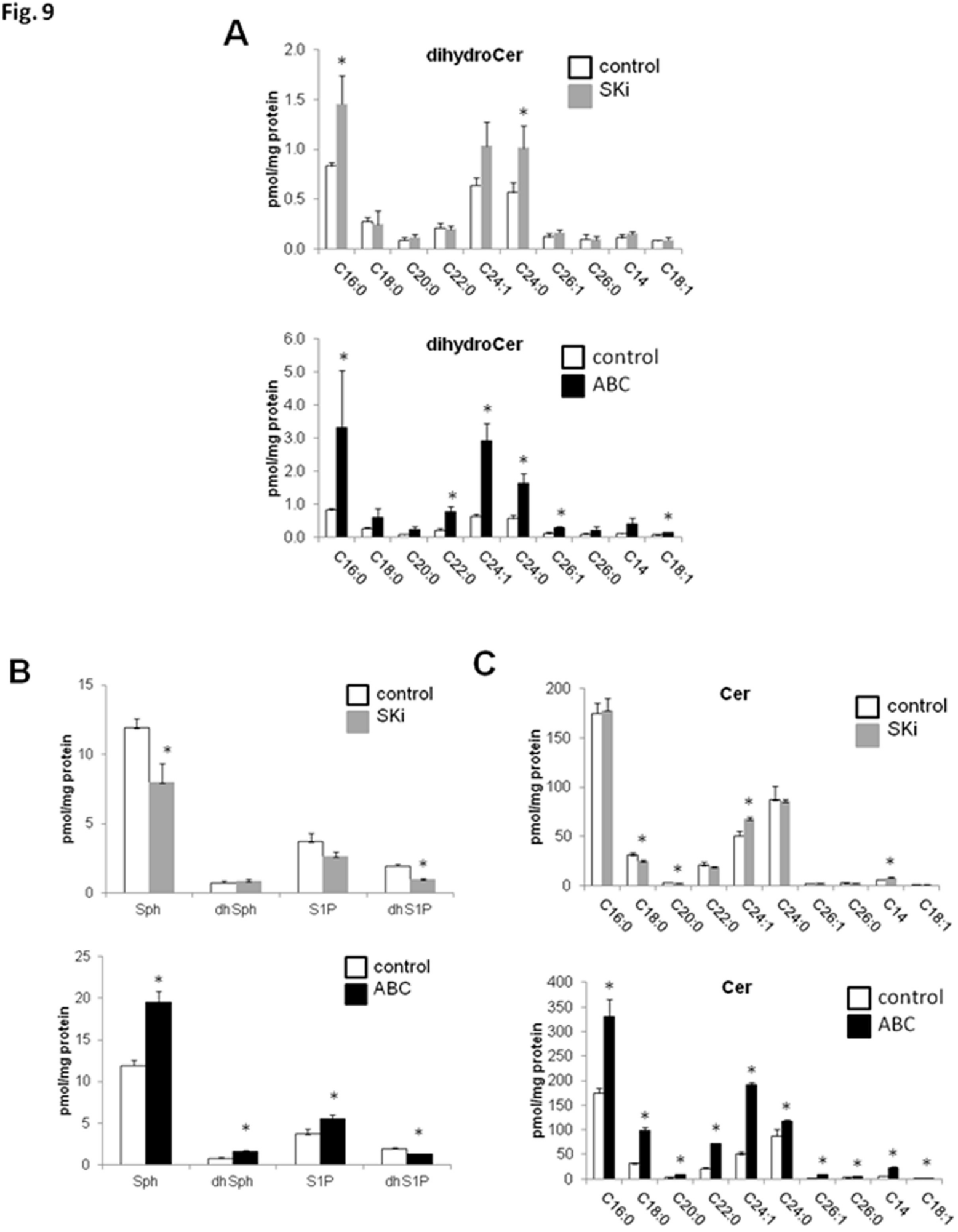
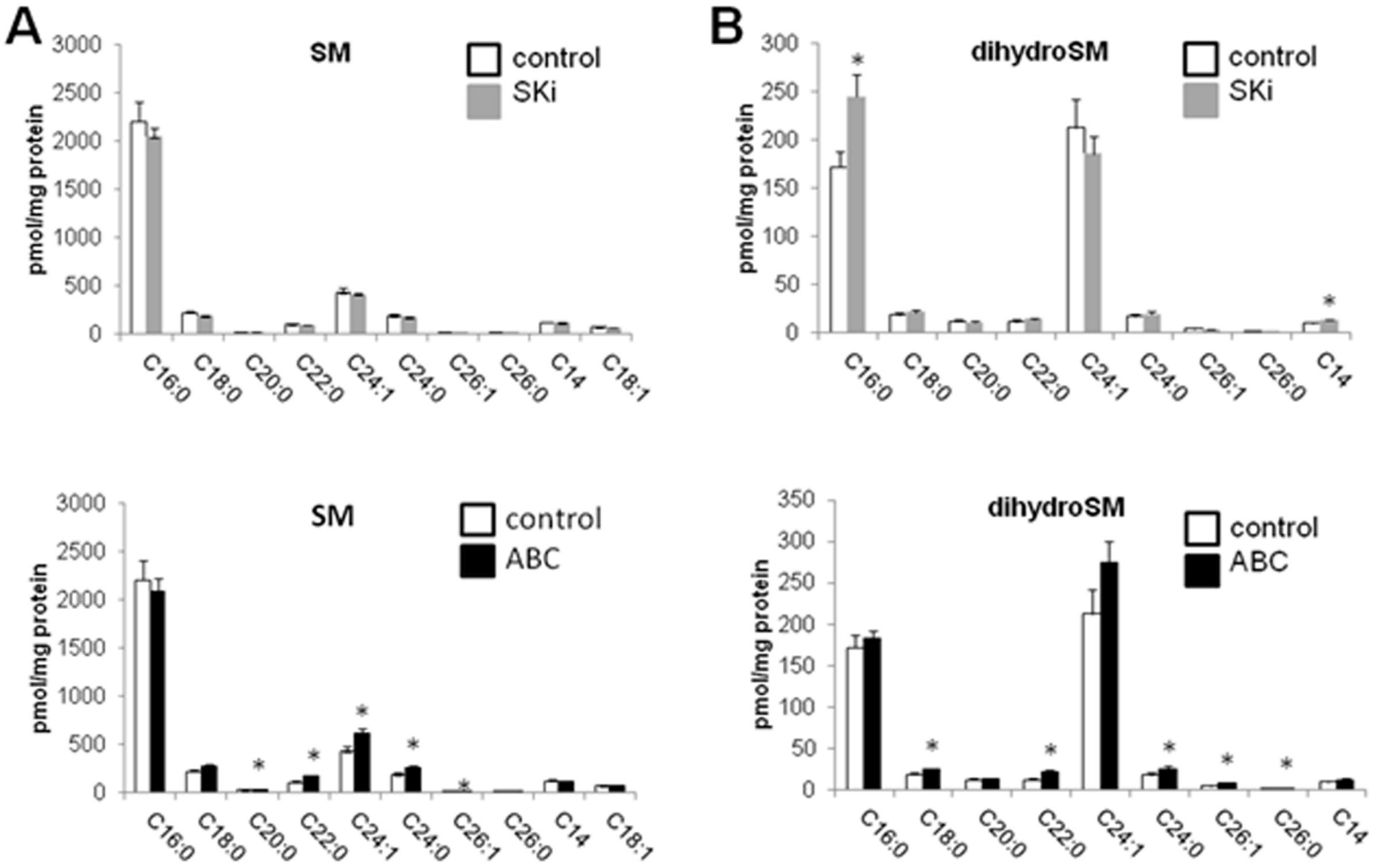

A
dihydroCer
control
SKi
pmol/mg protein
2.0
1.5
1.0
0.5
0.0
C16:0
C18:0
C20:0
C22:0
C24:1
C24:0
C26:1
C26:0
C14
C18:1
dihydroCer
control
ABC
pmol/mg protein
6.0
5.0
4.0
3.0
2.0
1.0
0.0
B
control
SKi
pmol/mg protein
15
10
5
0
Sph
dhSph
S1P
dhS1P
control
ABC
pmol/mg protein
25
20
15
10
5
0
C
Cer
control
SKi
pmol/mg protein
200
150
100
50
0
C16:0
C18:0
C20:0
C22:0
C24:1
C24:0
C26:1
C26:0
C14
C18:1
Cer
control
ABC
pmol/mg protein
400
350
300
250
200
150
100
50
0

Fig. 10


A
SM
control
SKi
pmol/mg protein
3000
2500
2000
1500
1000
500
0
C16:0
C18:0
C20:0
C22:0
C24:1
C24:0
C26:1
C26:0
C14
C18:1
B
dihydroSM
control
SKi
pmol/mg protein
300
250
200
150
100
50
0
C16:0
C18:0
C20:0
C22:0
C24:1
C24:0
C26:1
C26:0
C14
SM
control
ABC
pmol/mg protein
3000
2500
2000
1500
1000
500
0
C16:0
C18:0
C20:0
C22:0
C24:1
C24:0
C26:1
C26:0
C14
C18:1
dihydroSM
control
ABC
pmol/mg protein
350
300
250
200
150
100
50
0
C16:0
C18:0
C20:0
C22:0
C24:1
C24:0
C26:1
C26:0
C14

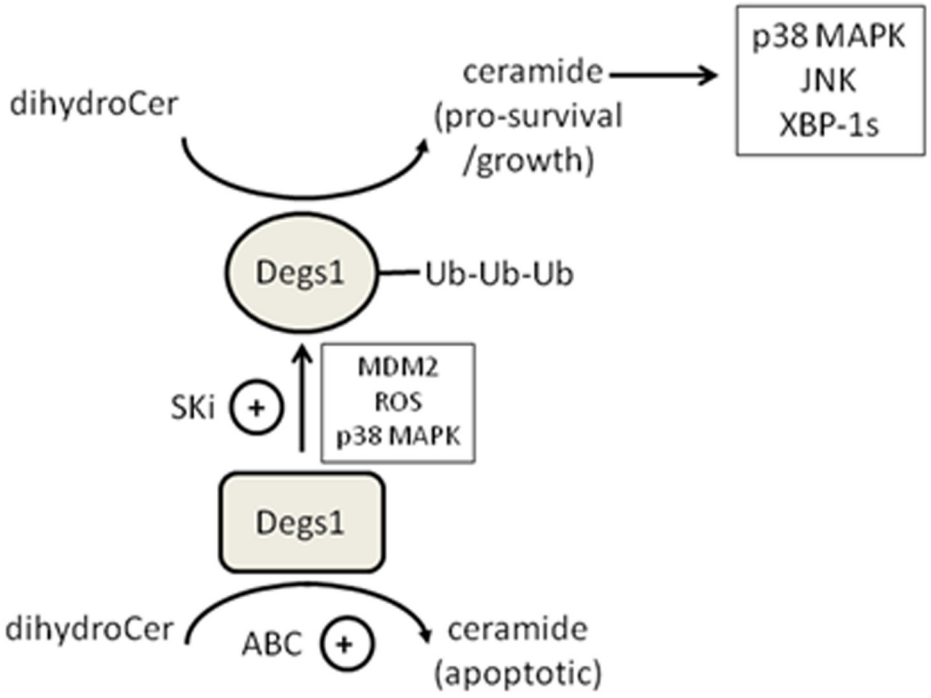

dihydroCer
ceramide
(pro-survival
/growth)
p38 MAPK
JNK
XBP-1s
Degs1
Ub-Ub-Ub
SKi
+
MDM2
ROS
p38 MAPK
Degs1
dihydroCer
ABC
+
ceramide
(apoptotic)

Fig. 11