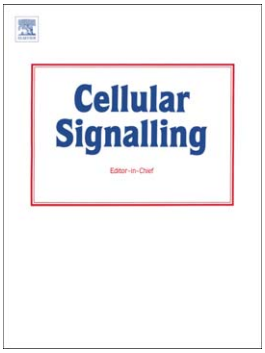

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RIG-I inhibits the MAPK-dependent proliferation of BRAF mutant melanoma cells via MKP-1

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Running title: RIG-I inhibits melanoma proliferation through MKP-1

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ABSTRACT

Background:BRAF-mutant melanoma is characterized by aggresive metastatic potential and therapeutic resistance. The innate immune receptor RIG-I has emerged as a potential target in melanoma therapies but the contributing pathways involved in anti-cancer activity are poorly characterized.

Methods: Baseline and ATRA-induced expression of RIG-I in nine (3 wild type and 6 BRAF-mutant) melanoma cell lines was measured with Q-PCR and Western blot. Ligand-specific stimulation of RIG-I was detected by Q-PCR and ELISA. Activation of the RIG-I-coupled IRF3, NF-κB and MAPK pathways was tested with protein array and Western blot. Cell proliferation and apoptosis was monitored by flow cytometry and cell counting. Down modulation of MKP-1 expression in melanoma cells was performed by specific siRNA.

Results: Short-term ATRA pre-treatment increases the expression of RIG-I in BRAF-mutant melanoma cells. Specific activation of RIG-I by 5'ppp-dsRNA leads to increased activity of the IRF3-IFNβ pathway but does not influence NF-κB signaling. RIG-I mediates the targeted dephosphorylation of several MAPKs (p38, RSK1, GSK-3α/β, HSP27) via the endogenous regulator MKP-1 resulting in decreased melanoma cell proliferation.

Conclusion: RIG-I has the potential to exert anticancer activity in BRAF-mutant melanoma via controlling IFNβ production and MAPK signaling. This is the first study showing that RIG-I activation results in MKP-1-mediated inhibition of cell proliferation via controlling the p38-HSP27, c-Jun and rpS6 pathways thus identifying RIG-I and MKP-1 as novel and promising therapeutical targets.

Keywords: RIG-I, MAPK, MKP-1/DUSP1, BRAF, melanoma, proliferation

1. INTRODUCTION

Melanoma is a malignant tumor of melanocytes that is responsible for the majority of skin cancer-related deaths. Because of its rapidly icreasing incidence and the lack of effective treatment, melanoma poses a serious threat worldwide.[1] Activating mutations in the serine-threonine protein kinase B-RAF (BRAF) are detected in the majority of patients with advanced melanoma exhibiting rapid tumor growth, increased metastatic capacity and significant therapeutic resistance.[2, 3]

Pattern recognition receptors (PRRs) are evolutionally conserved innate immune sensors expressed by many cell types. The recognition of specific molecular patterns by PRRs leads to the activation of complex signaling cascades eliciting type I interferon (IFN), inflammatory and chemokine responses. These immune responses depend on the delicate balance of the activation kinetics and cross-talk of different PRRs.[4, 5]RIG-I-like receptors (RLRs) are important PRRs that mediate interferon regulatory factor (IRF)-dependent IFN, and nuclear factor (NF)-κB-dependent inflammatory and chemokine responses, respectively.[6]The RLR-mediated production of type I IFNs depends on the phosphorylation and the subsequent nuclear translocation of IRF3. IRF3-associated signaling represents an early and rapid innate response that induces IFNβ production.The NF-κB pathway controlsthe production of inflammatory cytokines and chemokines including IL-6, CXCL8/IL-8 and CXCL10, which modulate the activation and migration of several immune cell types in the skin and other peripheral tissues.[7] RIG-I has recently emerged as a potential target for PRR-based molecular therapies in melanoma.[8-10]In these studies the specific activation of RIG-I and the RLR-mediated pathways were shown to trigger apoptotic cell death of melanoma cells in various settings.Furthermore, RIG-I-mediated apoptosis was reported to be associated with Bcl-2 and/or caspase-9 and Apaf-1 death pathways.[8, 9]However, other RLR-coupled

regulatory circuits involved in the development and biology of melanoma have still remained poorly characterized.

Mitogen-activated protein kinases (MAPKs) are key coordinators of many cellular processes. The classical MAPK families involve Extracellular Signal-regulated Kinases (ERKs), the p38 isoforms (p38α, p38β, p38γ and p38δ) and Jun N-terminal kinases (JNKs). They control or become modulated by other factors involved in cell proliferation and apoptosis, such as the Ribosomalprotein S6 kinases (RSKs), Glycogen synthasekinase 3α and β (GSK-3α/β) and Heat shock protein 27 (HSP27).[11, 12]Targeting MAPKs in chemotherapy-resistant BRAF mutant melanoma is an emerging and promising therapeutic approach,[2, 13]which can be achieved through the TNF receptor associated factor 6 (TRAF6) protein by directly triggering PRRs including Toll-like receptors (TLRs) and RIG-I.[11] The Mitogen-Activated Protein Kinase Phosphatase 1 (MKP-1; alsoknown as DUSP1) isan endogenous inhibitor of several MAPKs, including p38, Erk and JNKs.[11, 14]In early studies MKP-1 has also been described as a possible tumor suppressor gene with low constitutive expression in melanoma bearing BRAF mutation.[15, 16]Thus MKP-1 was identified as a possible key factor in drug-resistant tumors[17] however, the nature of its regulatory potential in melanoma biology has not been investigated so far.

In this study we aimed to examine the effects of RIG-I activation on the signaling events of NF-κB, IRF3 and various MAPK pathways in BRAF mutant melanoma cells. A particular interest was given to the RIG-I-mediated activation of MKP-1 and the consequences of MAP kinase phosphorylation, cellular death and proliferation. Our data demonstrate that the specific activation of RIG-I can effectively inhibit melanoma cell proliferation via MAPK pathways (p38, RSK1, GSK-3α/β, HSP27) and MKP-1 plays a crucial role in this process. These findings, by exposing novel pharmaceutical targets, may help to improve the efficacy of future melanoma therapies.

2. MATERIALS AND METHODS

2.1 Culturing and activation of melanoma cells

Wild type (M24met, MEWO, WM3060) and BRAF mutant (WM35, WM239, A2058, A375, WM3670, WM983A)human melanoma cells were obtained from ATCC (Teddington, Middlesex, UK) and the Wistar Institute(Philadelphia, PA), and cultured according to the recommended protocol. All-trans retinoic acid (ATRA) (Sigma, Schnelldorf, Germany)was used at a concentration of 1 μM in the assaysunless stated otherwise. The specific RIG-I ligand 5'ppp-dsRNA (InvivoGen, San Diego, CA) was used at working concentrations of 0.1-1 μg/ml. The transfection of 5'ppp-dsRNA was performed with the LyoVec system (InvivoGen) according to the manufacturer's protocol. To prepare cell lysates for Western blotting, the cells were activated for 15min – 24h as indicated in the figure legends. To collect supernatants for ELISA and prepare cell lysates for Q-PCR, sample collection was performed after 24 and 12h of activation, respectively.

2.2 RNA isolation, cDNA synthesis and QPCR

Real-time quantitative polymerase chain reaction (QPCR) was performed as described previously.[18] Briefly, total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA). 1.5-2 μg of total RNA was reverse transcribed using SuperScript II RNase H reverse transcriptase (Invitrogen) and Oligo(dT)15 primers (Promega, Madison, WI). Gene-specific TaqMan assays (Applied Biosystems, Foster City, CA) were used to perform QPCR in a final volume of 12 μl in triplicates using AmpliTaq Gold DNA polymerase and ABI StepOnePlus real-time PCR instrument (Applied Biosystems). Amplification of 36B4 was used as a normalizing control. Cycle threshold values (Ct) were determined using StepOne 2.1

software. Constant threshold values were set for each gene throughout the study. The sequence of the primers and probes are available upon request.

2.3 Cytokine measurements

Culture supernatants were harvested 24 hours after melanoma cell activation and the concentration of IL-6 cytokine was measured using OptEIA kits (BD Biosciences, San Jose, CA). The level of secreted IFNβ was measured by a Human Interferon beta ELISA Kit (Cell Sciences, Canton, MA).

2.4 Western blotting

Cells were lysed in Laemmli buffer and the protein extracts were tested by specific Ab recognizing RIG-I, IRF3, pIRF3, p65, pp65, HSP27, pHSP27, p38, pp38, c-Jun, pc-Jun (Ser63), rpS6, prpS6 (Cell Signaling, Danvers, MA, USA), MKP-1 (Santa Cruz, Dallas, TX) and β-actin (Sigma) all diluted 1:1000. The secondary Ab were used at a dilution of 1:5000. Anti-rabbit or anti-mouse Ab conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont Buckinghamshire, UK) were used as secondary Abs. The SuperSignal enhanced chemiluminescence system was used for probing target proteins (Thermo Scientific, Rockford, IL). After the membranes had been probed for RIG-I, IRF3, p65, MKP-1 or phospho-MAPKs, they were stripped and re-probed for β-actin or native MAPKs.

2.5 Phospho-MAPK protein array

Human Phospho MAPK array kit was purchased from R&D Systems (Minneapolis, MN). Cells were seeded into 6-well plates at a density of 10^6 cells per ml. After 50 minutes of activation the cells were rinsed with PBS and lysed with the provided buffer. A mixture of cell lysates and the provided phospho-specific antibody cocktail were incubated with each

human phospho-MAPK array overnight at 4ºC. After repeated wash steps the arrays were exposed to chemiluminescent reagent, and the nitrocellulose membranes were exposed to X-ray films. Phospho-MAPK array spot signals were developed on X-ray films and quantitated by scanning the film and analyzing the array image file using the image analysis software Kodak 1D 3.6. The relative expression levels of phosphorylated events were calculated by normalizing with the positive control signal intensities.

2.6 Cell proliferation assays

Cell proliferation was assessed by a Cell Counting Kit (CCK)-8 kit (Dojindo, Kumamoto, Japan). Briefly, 1×10^4 cells were seeded into 96-well plates and sampling was carried out after 24, 48 and 72 hours after ATRA and/or 5'ppp-dsRNA treatment. 15 mL CCK-8 reagent was added to each well 1 hour before the endpoint of incubation. The optical density (OD) values measured at 450 nm were determined by a microplate reader.

For thymidine incorporation assay, 1×10^4/ml cells were seeded in a 96-well culture plate in triplicates. 1.5 microcurie tritiated thymidine (3H-TdR; 1.5 μCi/well) was added to each well 6 h prior to the end of the culturing phase. The incorporation process was stopped with cold PBS solution. After digestion with 0.25% trypsin cells were collected on a glass fiber filter. Physiological saline (stabilized with 10% trichloroacetic acid) was used for washing the cells five times followed by destaining with absolute ethanol, and drying for 20 min at 65°C. Samples were subsequently transferred into scintillation fluid, and counted in a liquid scintillation counter (counts/min, cpm).

2.7 Cytotoxicity assays

The percentage of apoptotic cells was assessed by using an Annexin V apoptosis kit (BioVision, CA, USA) following the manufacturer's recommendations.The rateof necrotic

cell death was determined by measuring membrane integrity. The rate of necrotic cell death was quantified based on the loss of membrane integrity and the uptake of propidium iodide (PI). Cells were stained with 10 μg/ml PI before analysis by flow cytometry.For monitoring caspase-3 activity in intact cells the caspase-3/7 activity assay using a cell-permeable luminencence substrate (G811A) was used according to the manufacturer's recommendations (Promega). A total of 0.5×10^6 cells in 1 ml were activated for 24 h following stimulation, cells were plated and labeled with caspase-3/7 substrate. The data were analyzedby luminescence reader. DNA fragmentation, a charactheristic marker of apoptosis, was quantified by measuring the sub-G1 population. Cells were fixed in ice-cold 70% ethanol, washed in 38 mM citrate buffer (pH 7.4), and incubated for 20 min in 38 mM citrate supplemented with 50 μg/ml PI and 5 μg/ml RNase A (both from Sigma). Cells were analyzed with flow cytometry, and the proportion of sub-G1 particles with decreased DNA content was determined. For membrane integrity measurement, total cell death was quantified based on the PI uptake due to the loss of membrane integrity. Upon 24h stimulation the cells were harvested and stained with PI (10 μg/ ml) and analyzedimmediately by flow cytometry.

2.8 RNA interference

Gene-specific siRNA knockdownwas performed by SilencerSelect siRNA (Applied Biosystems) transfection using Gene Pulser Xcell instrument (Bio-Rad, Hercules, CA). Silencing of MKP-1 gene was performed by using a mix of the two available DUSP1/MKP-1 siRNAs. Silencer negative control nontargeting siRNA (Applied Biosystems) was used as a negative control. The efficacy of siRNA treatments was tested two days post-transfection by Western blotting.

2.9 Migration assay

Cells were suspended in migration medium(0.5 % BSA in RPMI 1640) at 10^6 cells/ml. Transwell migration inserts (diameter 6.5 mm; pore size 5 µm)were obtained from Corning (Lowell, MA). When spontaneous trans-endothelial migration assayswere performed the migration medium in the lower chamber wasRPMI 1640 (supplemented with 0.5 % BSA). Cells were addedto the upper chamber in a final volume of 250 µl andthe chemotaxis assays were conducted for 24 h in 5% CO_2 at 37^{o}C.At the end of the assay, the inserts werediscarded and cells that migrated to the lower chamber were collected. Migrated cell numbers were counted by flow cytometry using polystyrene standard beads (Transwell Migration Assay kit).

2.10Statistical analysis

Data are presented as mean ± SEM. A *t-test* was used for comparison of two groups. One-way ANOVA, followed by Bonferroni post hoc test, was used for multiple comparisons.Differences were considered to be statistically significant at p values < 0.05 (*).

3. RESULTS

3.1 Baseline and ATRA-induced expression of RIG-I in wild type and BRAF-mutant melanoma cell lines

BRAF mutant melanomas are notoriously resistant to most of the available chemotherapies. Recent studies have demonstrated that this resistance can be flanked by triggering innate PRRs, such as RIG-I.[8-10] Being a retinoic acid-induced gene, the expression of RIG-I can be strongly increased by ATRA, an active metabolite of vitamin A, which has been used as an adjuvant in melanoma chemotherapy.[19]The expression levels of RLRs in differenttypes of melanoma tumors have already been examined.[9]However, not all melanoma cells expressfunctional PRR proteins available for targeting.[20]Thus, we first examined the baseline gene expression levels of RIG-I innine different melanoma cell lines (3 wild type and 6 BRAF-mutant). We found that RIG-I is expressed in both wild type and mutant cells, however BRAF-mutant cell lines (WM35, WM239, A2058, A375, WM3670, WM983A) exhibited higher mRNA levels of this PRR as compared to the non-BRAF-mutant cells (M24met, MEWO, WM3060) (Figure 1A). Out of the six examined BRAF-mutant types, WM983A showed the highest RIG-I expression, therefore we chose this cell line for further investigations. We found that WM983A cells express RIG-I at both the mRNA and protein levels (Figure 1) and its expression could be further increased with 1 μM ATRA treatment. RIG-I transcript levels peaked after 12 h of ATRA stimulation (Figure 1A) and the highest protein expression could be achieved at 12 and 24 h post-treatment (Figure 1B). Statistical analysis of data from four independent experiments showed no significant difference in the levels of RIG-I protein at 12 h and 24 h after treatment (Figure 1B). Thus, in our further experiments we used a single 12-h ATRA pre-treatment to modulate the expression of RIG-I in melanoma.

3.2 Cytokine profile of melanoma cells is modulated by ligand-specific RIG-I stimulation

To check whether RIG-I is functional in the BRAF mutant WM983A cell line, we triggered this PRR with the highly specific ligand 5'ppp-dsRNA.[21] We found that the activation of melanoma cells by 5'ppp-dsRNA resulted in a marked increase in both the mRNA (Figure 2A) and secreted levels of IFNβ (Figure 2B). Furthermore, a 12-h ATRA pre-treatment applied before 5'ppp-dsRNA stimulation could increase the production of this cytokine significantly as compared to the ATRA non-treated cells (Figure 2). Interestingly, neither the mRNA levels (Figure 2A) nor the production of the inflammatory cytokine IL-6 (Figure 2B)could be modulated by this treatment. Similarly, no changes in the expression of chemokines IL-8 and CXCL10 were observed in stimulated WM983A cells (Figure 2A). In control experiments, ATRA alone was not able to cause any detectable alterations in the secretion levels of the measured cytokines within this time frame (Figure 2B).

3.3 RIG-I mediatesIRF3but not NF-κB signaling in melanoma cells

Since the detailed mechanism of RIG-I-induced IRF3 and NF-κB signaling has not been mapped yet in BRAF mutant melanomas, we next sought to test the activation of these pathways in WM983A cells. In accordance with our cytokine data (Figure 2) we observed increased phosphorylation of IRF3 following stimulation by 5'ppp-dsRNA and detected significantly elevated phospho-IRF3 levels in consecutively (ATRA+5'ppp-dsRNA) treated cells as compared to those activated with a single 5'ppp-dsRNA stimulation (Figure 3A). On the contrary, we found consistently high levels of phospho-p65 in these cells indicating a persistent activation of NF-κB pathway that could not be modulated by either 5'ppp-dsRNA treatment or combined stimulation with ATRA and 5'ppp-dsRNA (Figure 3B). We also did not observe significant changes of IRF3 or p65 phosphorylation in cells treated with ATRA only (data not shown). These findings altogether demonstrate that in WM983A cells RIG-I

specifically controls the IRF3-IFNβ axis but has no effect on the NF-κB pathway (Figures 2 and 3).

3.4 RIG-I induces dephosphorylation of multiple MAPKs in melanoma cells

To determine whether RIG-I could modulate the activation of MAPKs, a phospho-protein array including 26 kinases was performed with WM983A cell lysates obtained 60 min after stimulation with 5'ppp-dsRNA (Figure 4 and FigureS1). We found that the constitutive phosphorylation of four factors, such as HSP27, p38γ, RSK1 and GSK-3α/β was partially or completely inhibited by RIG-I activation. The influence of ATRA on this phenomenon seemed to be negligible as upon ATRA pre-conditioning only the rate of inhibition exerted on GSK-3α/β phosphorylation increased significanlty (Figure 4).Since the sensitivity of the MAPK array was below the treshold to discern and follow changes in the levels of these selected factors we performed focused Western blot analyses to dissect the phosphorylation patterns of HSP27, p38γ, RSK1 and GSK-3α/β in RIG-I stimulated cells (Figure 5). We found strong inhibition of the phosphorylation of HSP27 and p38 as well as that of c-Jun and ribosomal protein S6 (rpS6), known to be controlled by RSK1[22] and GSK-3α/β,[23] respectively. (Figure 5A). This inhibition occured within 2 h after RIG-I activation with a peak at 120 min pointing to an early regulatory mechanism. Interestingly, ATRA pre-treatment could enhance this inhibitory effect by decreasing the baseline level of HSP27 but not c-Jun phosphorylation (Figure 5A, 5B and 5D). RIG-I activation also decreased the levels of phosphorylatedp38 and rpS6 with no significant differences between ATRA treated versus non-treated cells (Figures 5A, 5C and 5E). Furthermore, the levels of p38 and HSP27 phosphorylation were mostly affected as early as 15 min after RIG-I ligation (Figures 5A, 5B and 5C).

3.5 RIG-I activation modulates cultured melanoma cell proliferation but not cell death

We showed that the phosphorylation/activation state of several MAPKs was negatively affected by RIG-I activation in WM983A cultures (Figure 5). Since it has been shown that GSK-3α/β, c-Jun, and the HSP27-p38-AKT axis are highly involved in the control of cellular proliferation and survival in melanoma and in other cancers, we next sought to determine the possible impact of RIG-I activation on melanoma cell growth and death.[24-26]5'ppp-dsRNA treatment caused a marked decrease in the proliferation of melanoma cells as compared to non-treated controls (Figure 6Aand Figure S2). This effect was not influenced by ATRA pre-conditioning as 5'ppp-dsRNA and ATRA+5'ppp-dsRNA treatments resulted in the same degree of inhibition of cellular proliferation (Figures 6A and S2).

To investigate whether attenuated proliferation is a consequence of the elevated ratio of cell death we examined the possible influence of RLR pathway activation on WM983A melanoma cell apoptosis and necrosis. Intriguingly, we could not observe either increased apoptosis or necrosis in the cell cultures following 5'ppp-dsRNA treatment alone or in combination with ATRA pre-treatment (Figures 6B-D). To check the possible involvement of other apoptotic pathways, caspase activity and DNA fragmentation simultaneously with membrane integrity were also measured but no cell death induction could be observed (Figures 6C-D). Sub-G1 populations never indicated more than 5% apoptosis (data not shown). We could not detect apoptosis even in 72 h upon RIG-I stimulation by Annexin staining (Figure 6B). Thus, RIG-I activation can significantly interfere with in vitro BRAF-mutant melanoma proliferation but not with cellular death.

3.6 RIG-I-mediated inhibition of MAPK signaling and melanoma cell proliferation is dependent on MKP-1

MKP-1 has been shown to selectively dephosphorylate and inhibit p38,[27] JNK/c-Jun,[28] ERK1/2-GSK-3,[29] and it has also been reported as a key endogenous suppressor of innate immune responses acting as a negative feedback regulator of MAPK activity.[30] Our results showing that the RIG-I-mediated dephosphorylation of HSP27, p38, c-Jun and rpS6 is an early event raised the possibility that an endogenous regulator with constitutive expression may play role in this phenomenon (Figures 4 and 5). To take a step further, we investigated the possible involvement of MKP-1 in this process by using a specific siRNA gene-silencing technology. Considering that ATRA had not influenced the effect of RIG-I activation on melanoma proliferation (Figure 6A, Figure S2), we did not perform ATRA pretreatments. Furthermore, as HSP27 is a downstream target of phosphorylated p38 (p38-HSP27 axis), and HSP27 contributes to cellular proliferation events in a p38-dependent manner,[31, 32] we therefore focused on the phosphorylation/activation of HSP27 in these experiments.

Gene-specific silencing resulted in 79 ± 3% (*n=3*) downregulation of MKP-1 protein in melanoma cells (Figure 7A). We observed the abscence of RIG-I-mediated inhibition of HSP27, c-Jun, and rpS6 in MKP-1 knock-down cells, while we found significant dephosphorylation of these MAPKs in non-targeting siRNA control-treated samples (Figure 7B). In accordance with these findings, the proliferation rate of 5'ppp-dsRNA+MKP-1 siRNA-treated cells did not differ significantly from the non-treated control cultures. On the other hand, 5'ppp-dsRNA treatment alone, or in combination with non-targeting control siRNA, resulted in a significant inhibition of cellular proliferation (Figure 7C). Additionally, specific MKP-1 knockdown did not lead to significant modulation of cellular proliferation *per se* (non-treated control versus MKP-1-negative cells; data not included).We observed a highly similar pattern of inhibition when tested three additional BRAF-mutant cell lines (WM35, WM239, and WM3670) that had previously been found to express high levels of RIG-I mRNA (Figure 1A and Figure 8). These results suggest a critical role of MKP-1 in the RIG-I-

mediated dephosphorylation and inhibition of HSP27-p38, c-Jun, and rpS6 leading to a marked decrease in the proliferation of BRAF-mutant melanoma cells.

4. DISCUSSION

BRAF-mutant melanomas exhibit an insurmountable obstacle in the clinical treatment of malignant skin cancers. Novel therapeutic strategies involve the mobilization of immune cells close to the tumor microenvironment by using cytokines as adjuvants.[33, 34]Novel approaches, such as the administration of type I IFNs, target both antigen-presenting cells (APCs) and cytotoxic T lymphocytes thus icreasing anticancer activitysignificantly.[1, 35] Type I interferonssuch as IFNβ is known as one of the major effector cytokinesthat couples innate and adaptive immunity and mediatesstrong anticancer responses by activating manydifferent cell types acting as possible effectors in novel molecular anti-tumor therapies.[36-38]The innate immune receptor RIG-I is known to control type I IFN production in many cell types and it has also been identified as a promising target in melanoma therapy.[8-10] Although the role of RIG-I in cellular death has already been characterized in these reports, the involvement of other RIG-I-coupled pathways in melanoma biology is yet to be understood.

In this study, we sought to identify the role of RIG-I and its downstream signaling pathways mediated by NF-κB, IRF3 and MAPKs in the regulation of melanoma cell apoptosis and proliferation. We report here that the BRAF-mutant melanoma cell lines WM35, WM239, A2058, A375, WM3670, and WM983A express higher baseline levels of RIG-I mRNA as compared to wild type (M24met, MEWO, WM3060) cultures (Figure 1A). This expression could be further enhanced with ATRA treatment, leading to a relatively early peak of RIG-I protein expression within 12 hours (Figure 1B). Specifically triggering RIG-I in in vitro WM983A cultures with 5’ppp-dsRNA resulted in the production of IFNβ that could be

significantly boosted with a 12h ATRA pre-treatment (Figure 2) in line with our previous findings demonstrating an increased expression of RIG-I receptor in ATRA pre-treated cells (Figure 1). These findings may reflect to a potent anticancer activity of RIG-I via the control of IFNβ production in melanoma, which can lead to the mobilization of nearby immune cells in the tumor microenvironment.[38]

Interestingly, the mRNA levels of inflammatory cytokines and chemokines did not change, irrespective of RIG-I activation and/or ATRA pre-conditioning (Figure 2A). Similarly, the levels of the secreted inflammatory cytokine IL-6 remained unchanged in melanoma cultures treated with 5'ppp-dsRNA and/or ATRA (Figure 2A and 2B). To trace the activity of the related pathways coupled to RIG-I, we next measured the levels of the phosphorylated transcription factors IRF3 and p65, which control the expression of IFNβ and inflammatory cytokines and chemokines, respectively. In accordance with our previous cytokine data (Figure 2), RIG-I stimulation led to the the activation of the IRF3 pathway that could be enhanced with ATRA pre-treatment (Figure 3A). The activation state of p65 (NF-κB pathway) remained unchanged independently of the applied stimuli (Figure 3B). This is in agreement with previous findings showing the continuous activation of NF-κB in BRAF mutant melanomas that contributes to increased cell survival capacity.[39, 40]

Targeting MAPKs in BRAF mutant melanoma through PRRs, such as RIG-I represents a novel and promising approach in cancer pharmacotherapy.[2, 11, 13] These approaches have focused on the apoptosis-resistance of melanoma cells. However, other aspects of RIG-I-mediated MAPK activation in melanoma has remained largely unexplored. We therefore investigated the consequences of RIG-I-coupled MAPK activation in BRAF mutant WM983A cells. Our phospho-MAPK protein array data revealed that specific RIG-I stimulation has the potential to alter the phosphorylation state of four kinases (Figure 4). The constitutive phosphorylation states of HSP27, p38γ, RSK1 and GSK-3α/β were partially or

completely inhibited by RIG-I activation and the influence of ATRA pre-treatment on this phenomenon was negligible, as only the rate of GSK-3α/β inhibition increased significantly upon ATRA pre-conditioning (Figure 4). To dissect the kinetics and detailed mechanisms of this inhibition, we performed focused Western blot analyses to determine the phosphorylation patterns of HSP27, p38γ, RSK1 and GSK-3α/β following RIG-I stimulation. We found that RIG-I activation strongly inhibits the phosphorylation of HSP27, p38, c-Jun (controlled by RSK1) and rpS6 (regulated by GSK-3α/β) (Figure 5A). Inhibition of the constitutive phosphorylation state occured within two hours post-RIG-I-activation suggesting the operation of an early regulatory mechanism. Our densitomery data also showed that brief (12 hours) ATRA pre-conditioning significantly increased this inhibitory effect on HSP27 (Figure 5B) but not on c-Jun phosphorylation (Figure 5D). As a result of ATRA pre-treatment the baseline level of phospho-c-Jun was elevated, however, RIG-I stimulation led to significant inhibition of baseline c-Jun phosphorylation regardless of this elevation (Figure 5D). We also found a marked RIG-I-mediated inhibition of constitutive p38 and rpS6 phosphorylation that was not influenced by ATRA pre-treatment (Figures 5C and 5E). Moreover, the phosphorylation of HSP27 and p38 was the mostly affected occuring 15 minutes after RIG-I stimulation (Figures 5A, 5B and 5C). This fits in the current paradigm as p38 and HSP27 are members of the same MAPK pathway, known as the p38-HSP27 axis, and are co-regulated in as much as HSP27 phosphorylation is controlled by p38.[31, 32]The RIG-I-to-MAPK effects have been observed to be relatively fast (15 to 120 minutes)and we presume that the long-term consequences of these effects in cellular functions are mostly depending on these early signaling events.

We found that RIG-I activation in melanoma cells resulted in a marked decrease in the otherwise constitutively highly phosphorylated state of several MAPKs involved in the regulation of cell cycle and apoptosis (Figures 4 and 5). Based on this finding we next

investigated whether RIG-I ligation could affect in vitro melanoma cell proliferation and/or the rate of cell death. Melanoma cells transfected with 5'ppp-dsRNA exhibited significantly lower proliferation rate as compared to the non-transfected, vehicle-transfected and ATRA-only controls, and a 12h ATRA pre-treatment did not influence this inhibition either (Figures 6Aand S2). As the RIG-I-mediated inhibition of MAPK phosphorylation did not affect p38 and rpS6, (Figure 5A, 5C and 5E), both involved more dominantly in cell cycle control than c-Jun and HSP27,[11] we conclude that the ATRA-independent anti-proliferative effect of RIG-I stimulation predominantly relies on p38 and rpS6. RIG-I activation has been reported to cause cellular death in melanoma cells by interfering with the expression and function of many factors including caspase-9, Bcl-2 and Apaf-1.[8, 9]However, these well-designed studies were performed with non-BRAF mutant melanomas. Thus, taking a further step, we sought to identify the possible consequences of RIG-I-mediated inhibition of MAPKs in the apoptotic events in the BRAF mutant melanoma cell line WM983A. Strikingly, we found that neither short nor long term RIG-I activation could elicit cellular death via apoptosis or necrosis in WM983A cells and no evidence of RIG-I ligation on this effect could be detected (Figure 6B, 6C and 6D). This is in line with our previous findings showing the constitutive activation state of the NF-κB pathway, known to be involved in melanoma apoptosis-resistance, which could not be influenced with any of our treatment options (Figure 3). Taken together, our results suggest that RIG-I activation downregulates cellular proliferation, but not apoptosis in BRAF mutant melanoma.

The endogenous MAPK regulator MKP-1 has been shown to selectively dephosphorylate and inhibit p38, c-Jun, ERK1/2-GSK-3 and can also suppress innate immune responses trough the negative control of several other MAPKs.[27-30]Since our results showed that the RIG-I-dependent dephosphorylation of p38, HSP27, c-Jun and rpS6 was an early event (Figures 4 and 5), we hypothesized that an endogenous, constitutively expressed regulator of MAPK

phosphorylation may stand in the background of this inhibition. To address this possibility, we tested whether MKP-1 was involved in this phenomenon by using the gene-specific siRNA technique (Figures 7 and 8). The RIG-I-mediated dephosphorylation/deactivation of c-Jun, rpS6 and the p38-HSP27 axis occured only in the control samples that had originally contained functional MKP-1. However, in the MKP-1 knock-down cultures RIG-I stimulation did not result in the dephosphorylation of MAPKs pinpointing the critical role of MKP-1 in this process (Figure 7B). In line with this, the proliferation rate of 5'ppp-dsRNA-treated MKP-1 knock-down cells was similar to that of the non-treated control samples. In contrast, specific RIG-I activation alone or in combination with non-targeting siRNA led to a significant inhibition of in vitro BRAF-mutant melanoma proliferation (Figure 7C and 8).

In summary, our data show for the first time that the role of MKP-1 is indispensable in the RIG-I-mediated negative control of p38-HSP27, c-Jun and rpS6 phosphorylation and in the consequent inhibition of melanoma cell proliferation. Novel pharmacotherapies of cancer involve the specific modulation of MAPKs and their regulatory pathways involved in cellular proliferation and differentiation.[41] MKP-1 was originally identified as a key factor in chemotherapy-resistant tumors[17] however, the nature of its regulatory capacity in melanoma biology has not been investigated before. Targeting innate immune receptors in melanoma represents a novel and attractive approach to interfere with cancer progression and metastasis.[42, 43]This is the first study showing that specific RIG-I activation leads to an MKP-1-mediated inhibition of melanoma proliferation via the negative control/dephosphorylation of p37-HSP27, c-Jun and rpS6. Thus, our results identify RIG-I and MKP-1 as promising targets of future therapies in BRAF-mutant melanoma.

5. ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1

Baseline and induced expression of RIG-I in melanoma cell lines

A: Baseline mRNA expression of RIG-I in various wild-type melanoma cell lines (empty bars) or cells harboring BRAF mutation (black bars). **B:** Relative mRNA expression of RIG-I in the BRAF-mutant WM983A cell line upon treatment with 10^{-6} M all-trans retinoic acid (ATRA). Results represent the Mean ± SEM of 3 (Fig1A) or 5 (Fig1B) independent experiments. **C:** Induction of RIG-I protein expression in WM983A cells measured by Western blot. Result of a typical experiment out of four is shown. Densitometry data show the Mean ± SEM of four independent measurements.

Figure 2.

Cytokine and chemokine profile of melanoma cells induced by RIG-I-specific activation

A: Relative gene expressions of IFNb1 and IL-6 cytokines and CXCL8/IL-8 and CXCL10 chemokines in melanoma cells tested after 12h treatment with the RIG-I-specific ligand 5'ppp-dsRNA (5'ppp). Results are shown as Mean ± SEM of triplicates of four independent

measurements. **B:**IFNβ and IL-6 production of melanoma cells. Grey columns represent WM983A cells stimulated with 0.1-1.0 μg/ml 5'ppp-dsRNA, black columns show cells pre-treated with ATRA (10^{-6} M, 12h) followed by stimulations with 0.1-1.0 μg/ml 5'ppp-dsRNA for 12h. ATRA-only (ATRA ctrl) and non-treated (ctrl) cells were used as controls. Results are shown as Mean ± SEM of duplicates of three independent experiments.*p* values are indicated as: * ($p< 0.05$), ** ($p< 0.01$), *** ($p< 0.005$).

Figure 3.

RIG-I-mediated type I IFN and NF-κB signaling in melanoma cells

Phosphorylation profile of IRF3 and NF-κB after RIG-I stimulation. Cells were activated with 1 μg/ml 5'ppp-dsRNA (5'ppp-dsRNA) for 0-120 minutes and cell lysates were prepared for Western blotting. Another fraction of melanoma cells was pre-treated with 10^{-6} M ATRA for 12h followed by activation with 5'ppp-dsRNA. **A:**RIG-I-mediated IRF3 phosphorylation in ATRA pre-treated and ATRA non-treated cells. A representative blot image of a typical experiment out of three is presented. **B:** p65 phosphorylation in 5'ppp-dsRNA-activated melanoma cells, using the same setup as described in Figure 1 and 2. (**A-B:**) Relative density values are presented as Mean ± SEM of three independent experiments. Asterisk indicates significance ($p<0.05$).

Figure 4.

RIG-I-mediated induction of MAPK phosphorylation in melanoma cells

WM983Amelanoma cells were triggered with RIG-I ligation and 50 minutes after induction the cells were lysed and the phosphorylation of MAPKs was detected and analyzed as described in the Materials and Methods. Relative density values of phosphorylated MAPKs in non-treated cells (ctrl), in cells treated with 10^{-6} M ATRA (ATRA) or 1 μg/ml 5'ppp-dsRNA

(5'ppp), and in ATRA pre-treated (10^{-6} M, 12h) cells followed by activation with 1µg/ml 5'ppp-dsRNA (ATRA+5'ppp).**A:** Array of the investigated kinases (phosphorylation chart). **B:**Relative density values and kinetics of MAPKs exhibiting the highest changes in the phosphorylation state over the activation period. Data are presented as Mean ± SEM of duplicates of two independent experiments. Asterisk indicates statistical significance (p<0.05).

Figure 5.

RIG-I activation interfereswith the phosphorylation of selected members of MAPKs

ATRA pre-treatment and 5'ppp-dsRNA activation of melanoma cells were performed as described in Figures 3 and 4. **A:** Phosphorylation level of MAPKs was measured by Western blot. Results of a representative experiment out of three are shown. **B:**Densitometry data of phospho-HSP27, phospho-p38, phospho-c-Jun and phospho-rpS6 blots. Data are presented as Mean ± SEM of three independent experiments. (*) represents p values < 0.05.

Figure 6.

The effect of RIG-I ligation on the proliferating capacity and cell death of melanoma cells

Proliferation and cytotoxicity assays were carried out as described in the Materials and Methods section. WM983A cells were activated either by 1 µg/ml 5'ppp-dsRNA (5'ppp-dsRNA) or were pre-treated with 10^{-6} M ATRA (12h) prior to RIG-I activation [5'ppp-dsRNA (ATRA-pre-treated)]. Non-treated melanoma cells (non treated), WM983A melanoma cells treated with Lyovec (vehicle ctrl) or 10^{-6} M ATRA (ATRA) were used as controls. **A:** Proliferation rate of melanoma cells measured by the CCK-8 kit. Results of three independent experiments are shown as Mean ± SEM. Asterisk indicates significance compared to

controls(p<0.05). **B:**Rate of melanoma cell death following RIG-I activation. Induction of apoptosis was evaluated by Annexin V-FITCstaining. Results of three independent experiments are shown as Mean ± SEM; *n.s.* refers to non-significant changes. **C-D:**Investigation of cell death in melanoma cells. WM983A cells were activated as before and after 24h the cells were harvested and loaded with caspase-3/7 substrate (C) or PI (D). Caspase activity measured by luminescence intensity and membrane integrity was analyzed by flow cytometry. Data represent the average of three independent experiments.

Figure 7.

The effect of MKP-1 gene silencing on RIG-I mediated MAPK dephosphorylation and melanoma cell proliferation

All experiments were performed with WM983A cells. siRNA knock-down of MKP-1 was performed as in Materials and Methods. NT: non-treated control; siRNA control: non-targeting negative control siRNA; MKP-1 siRNA: MKP-1/DUSP1-specific siRNA. **A:** Western blot validation of MKP-1 silencing. A typical experiment out of four is demonstrated. **B:**Effect of MKP-1 knock-down on RIG-I-dependent MAPK signaling. Cells were activated with 1µg/ml 5'ppp-dsRNA for 0-120 min. Densitometry data represent Mean ± SEM values of three independent experiments. Asterisk indicates statistical significance (p<0.05).**C:** Proliferation rate of melanoma cells measured by the CCK-8 kit. Results of three independent experiments are shown as Mean ± SEM. Asterisk indicates significance as compared to control siRNA (p<0.05).

Figure 8.

Effects of MKP-1 gene silencing on the RIG-I-modulated proliferation of BRAF-mutant melanoma cell lines

siRNA knock-down of MKP-1 was performed as in Methods and in Figure 7. WM35, WM239, and WM3670 BRAF-mutant melanoma cells were activated with 1μg/ml 5'ppp-dsRNA or left untreated (non-treated ctrl). Cellular proliferation was monitored as in Figure 7C. In each cases, data are presented as Mean ± SEM of three independent experiments. (*) represents significance as compared to control siRNA (p<0.05).

SUPPLEMENTARY FIGURES

Figure S1.

RIG-I-mediated induction of MAPK phosphorylation in melanoma cells

After RIG-I mediated induction (50 minutes) the melanoma cells were lysed and the phosphorylation of MAPKs was detected as described in Fig4. Relative density values of phosphorylated MAPKs in non-treated samples (ctrl) and in cells treated with 10^{-6} M ATRA (ATRA) or 1 μg/ml 5'ppp-dsRNA (5'ppp), and ATRA pre-treated cells (10^{-6} M, 12h)followed by1 μg/ml 5'ppp-dsRNA activation (ATRA+5'ppp) are shown.

Figure S2.

The effect of RIG-I ligation on the proliferation of melanoma cells.

Thymidine incorporation assay was carried out as described in Materials and Methods. Treatments of WM983A cells were performed as in Figure 6. Results of three independent experiments are shown as Mean ± SEM. Asterisk indicates significance compared to controls (p<0.05).

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Figure 1

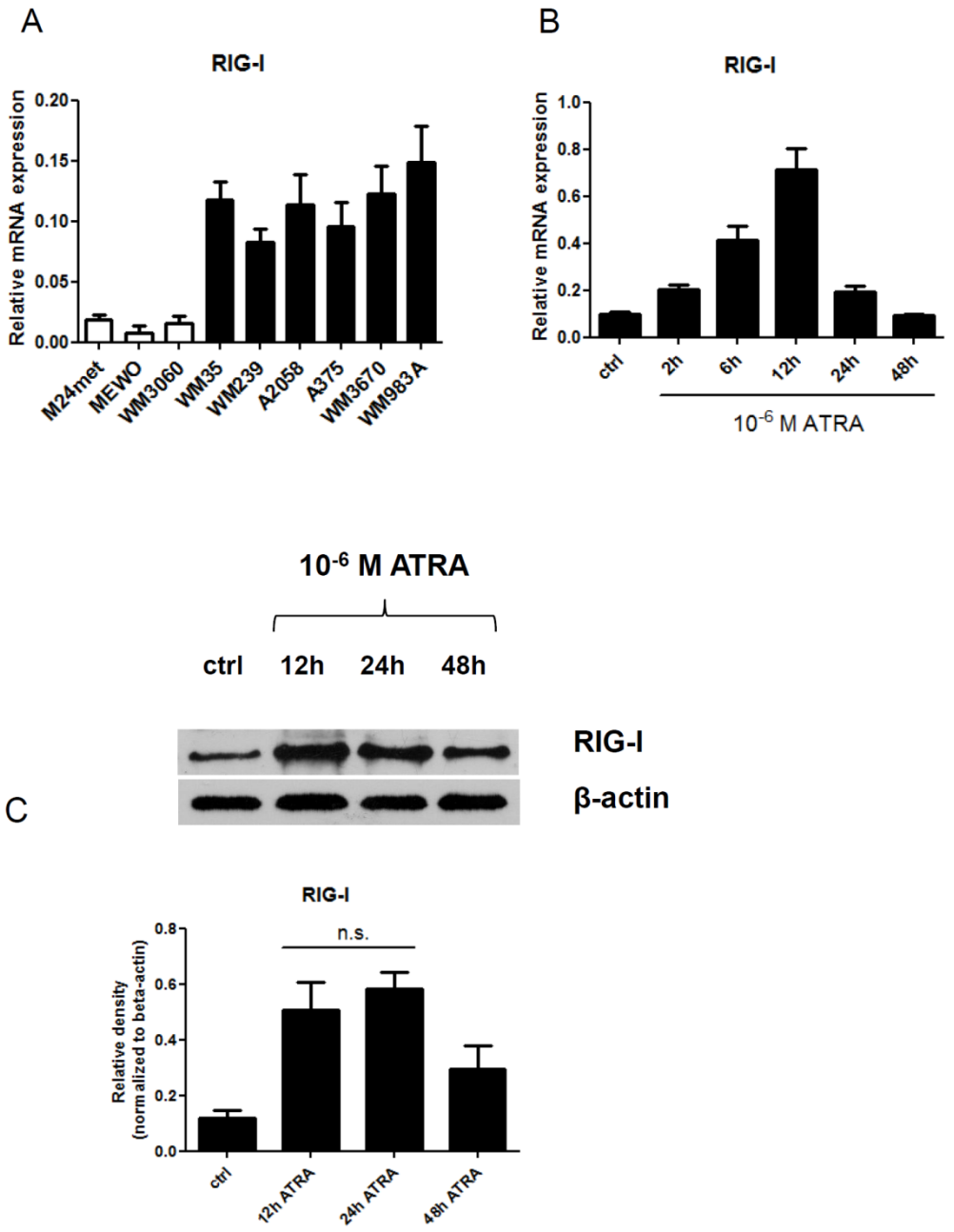

Figure 2

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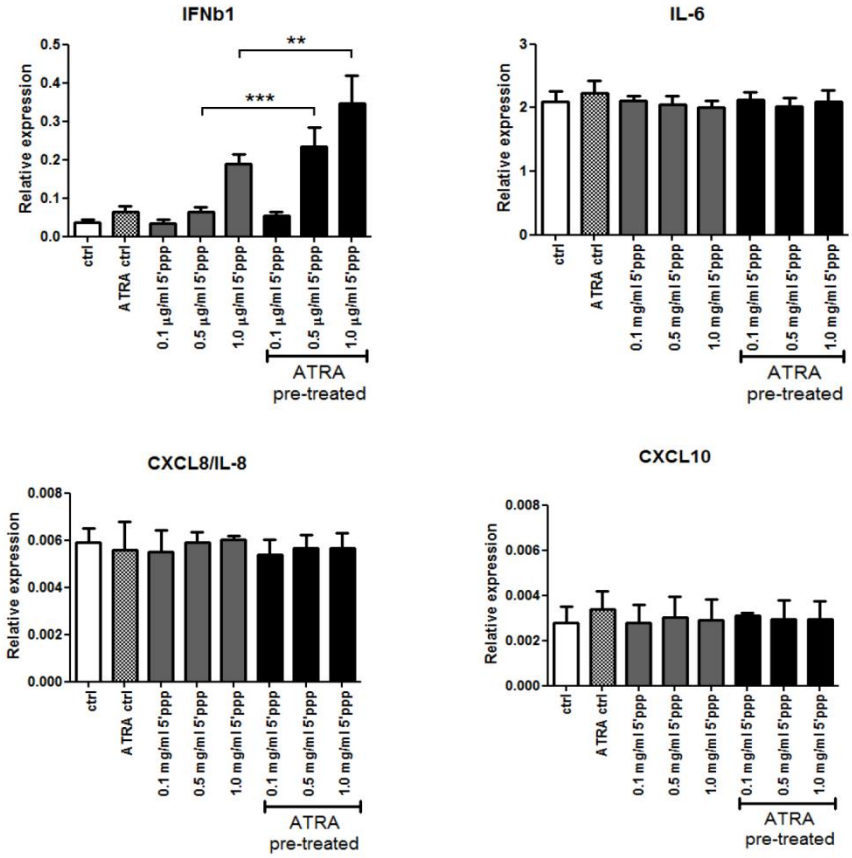

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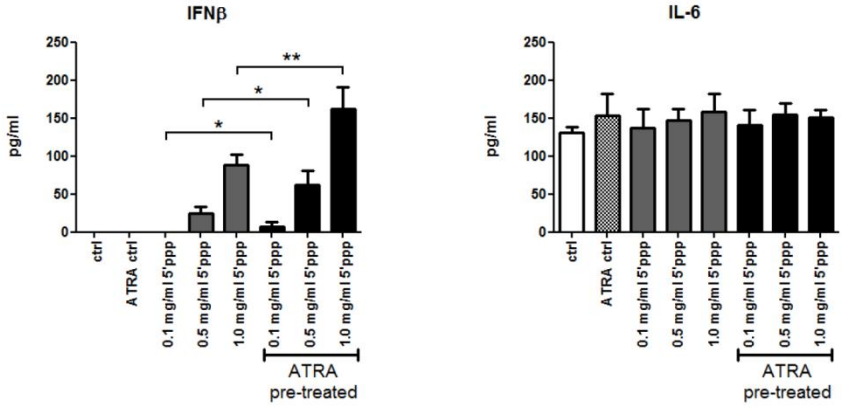

Figure 3

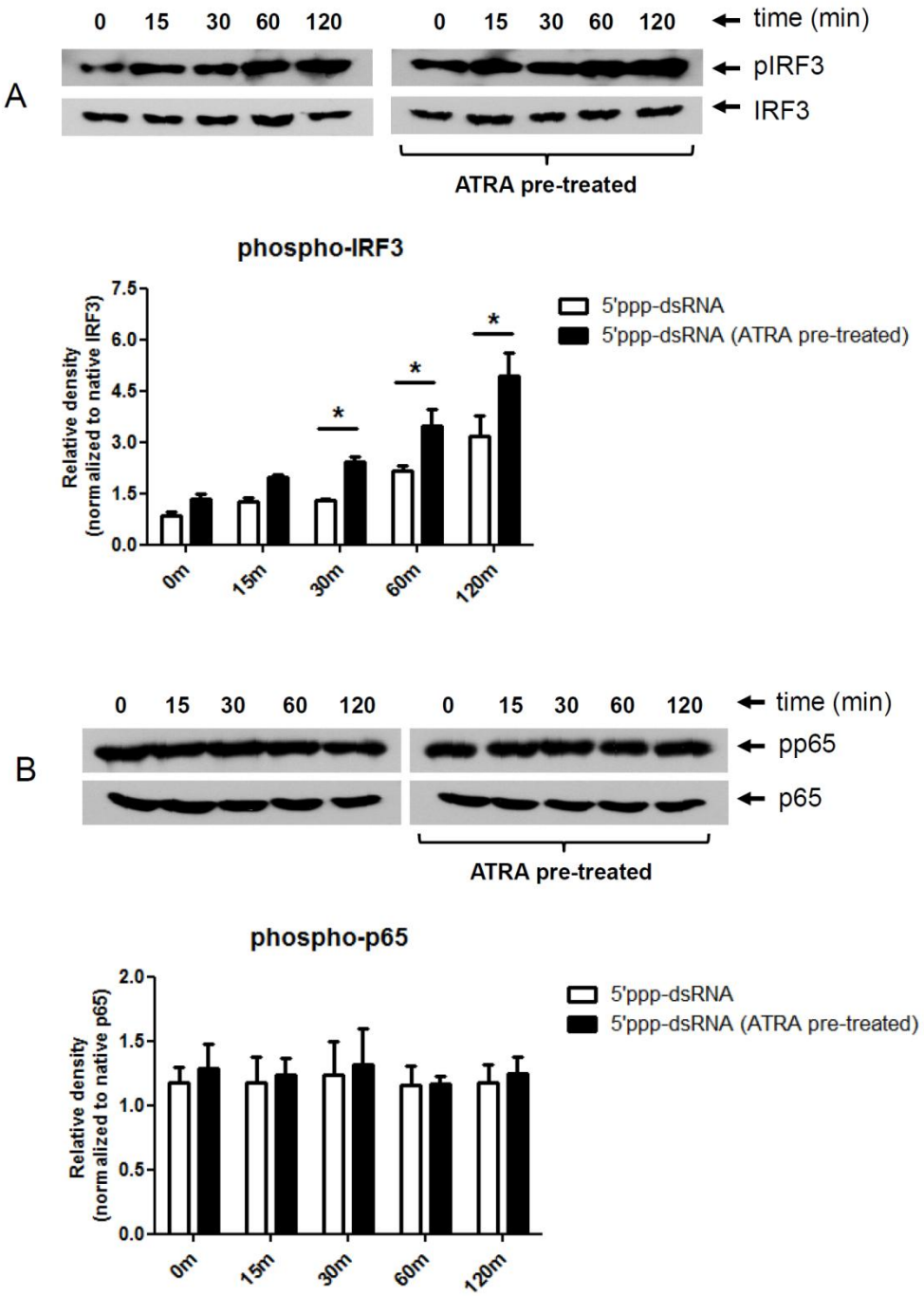

Figure 4

A

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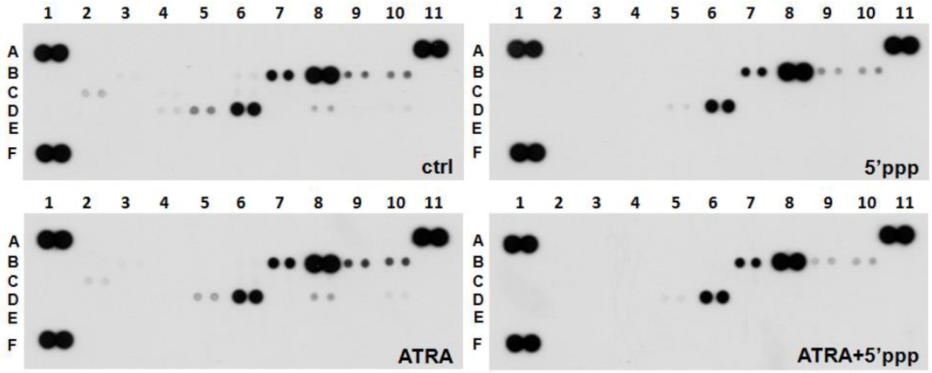
A	control (+)										control (+)
B		Akt1	Akt2	Akt3	Aktpan	CREB	ERK1	ERK2	GSK-3α/β	GSK-3β	
C		HSP27	JNK1	JNK2	JNK3	JNK pan	MKK3	MKK6	MSK2		
D		p38α	p38β	p38δ	p38γ	p53	p70 S6 kinase	RSK1	RSK2	TOR	
E										control (-)	
F	control (+)										



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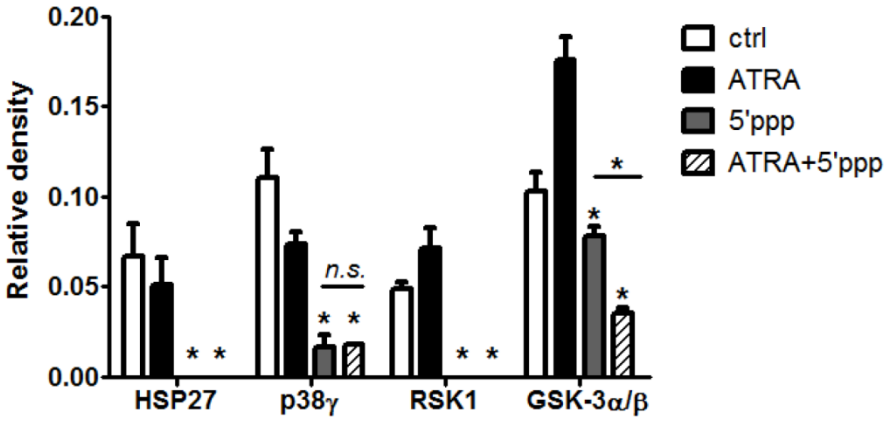


Figure 5

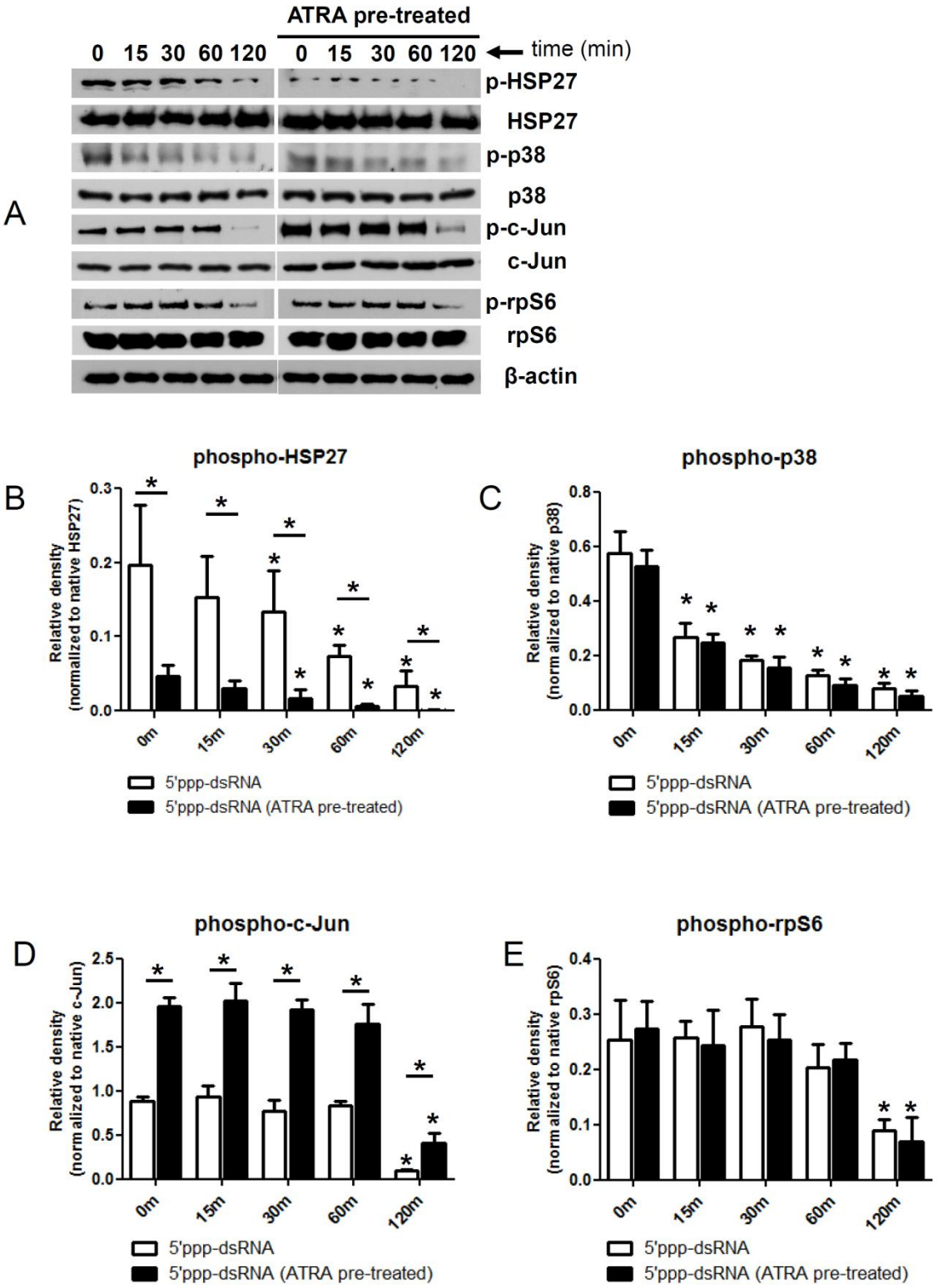


Figure 6

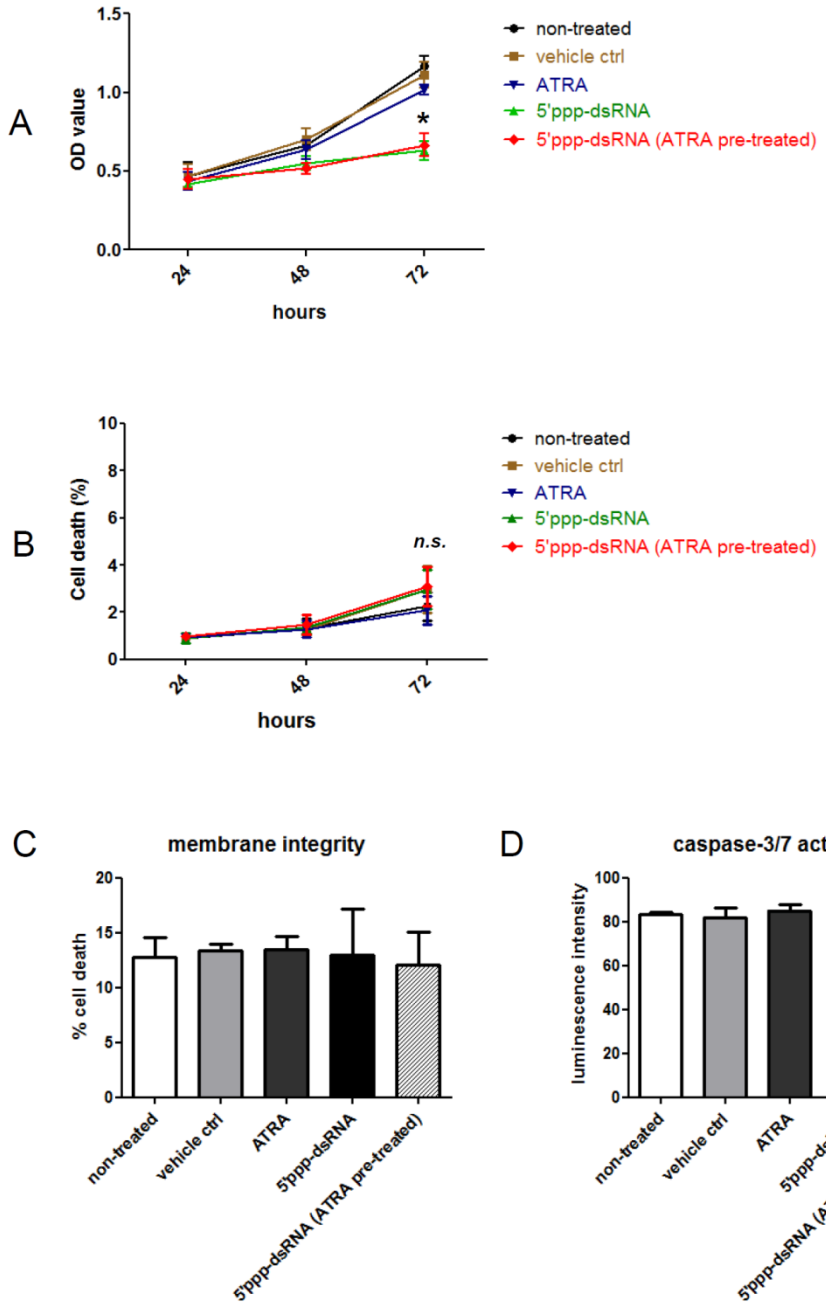


Figure 7

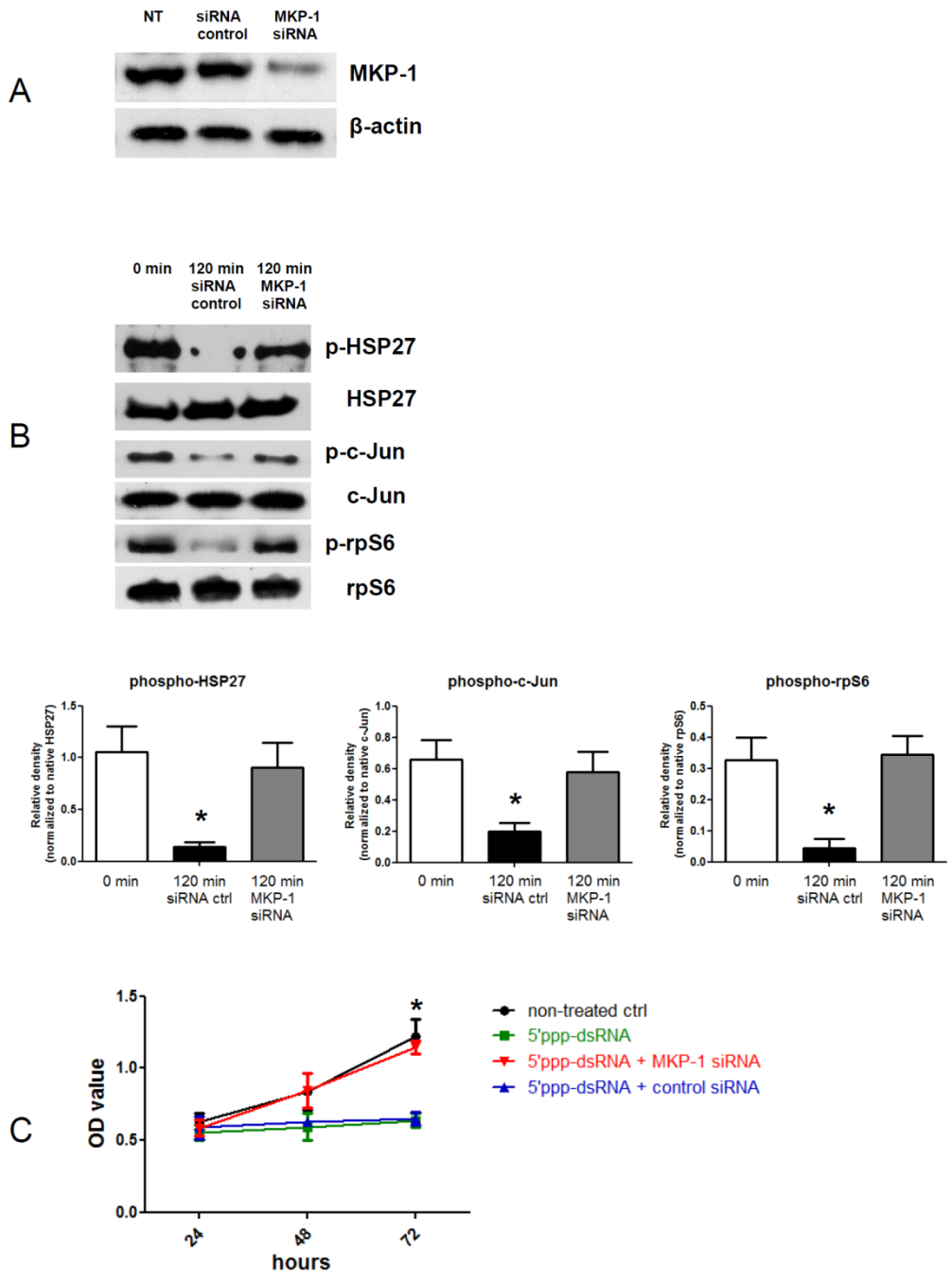
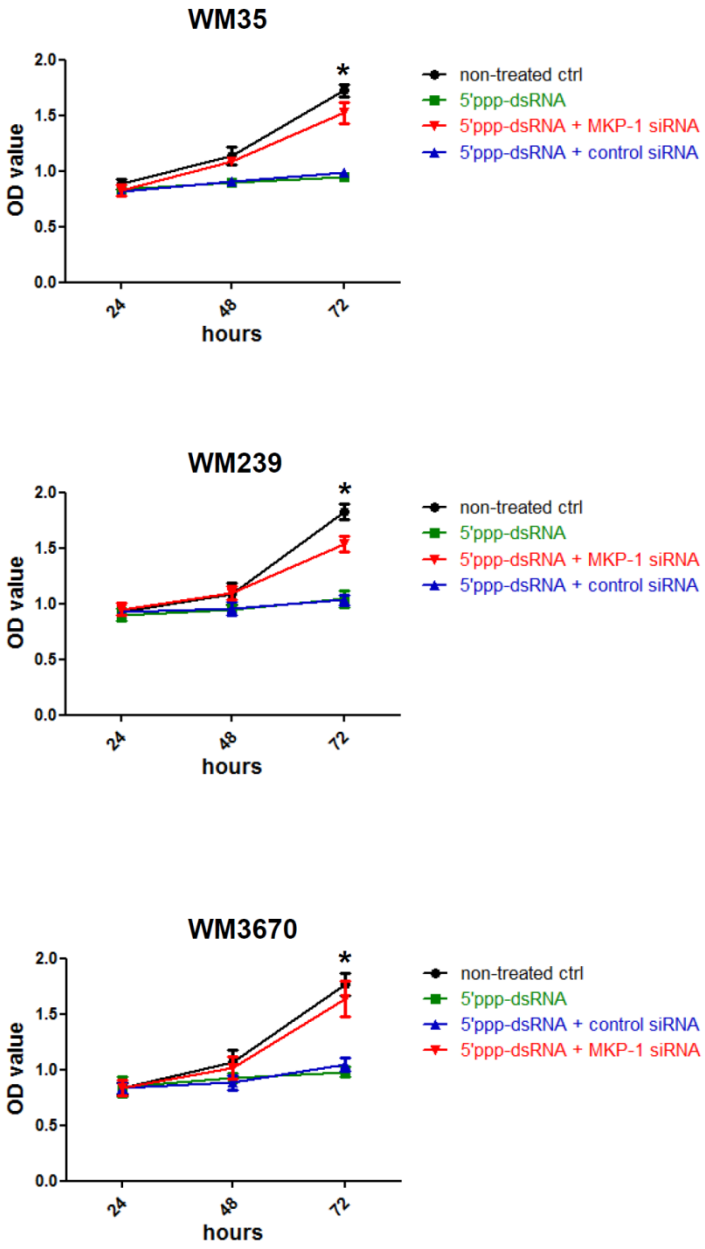


Figure 8



HIGHLIGHTS

- RIG-I exertsanticanceractivityinBRAF-mutantmelanomaviacontrollingIFNβproduction and MAPK signaling;

- RIG-I activationresultsin MKP-1-mediated cytostaticeffectinBRAF-mutantmelanoma;

- RIG-I/MKP-1-mediated inhibition of cellularproliferationinvolvesp38-HSP27, c-Jun and rpS6 MAPK pathways;