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Synchronized targeting of Notch and ERBB signaling suppresses melanoma tumor growth through inhibition of Notch1 and ERBB3*

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Abstract

Despite significant advances in melanoma therapy, melanoma remains the deadliest form of skin cancer, with a five-year survival of only 15%. Novel treatments are therefore required to address this disease. Notch and ERBB are evolutionarily conserved signaling cascades required for the maintenance of melanocyte precursors. We show that active Notch1 ($Notch1^{NIC}$) and active (phosphorylated) ERBB3 and ERBB2 correlate significantly and are similarly expressed in both mutated and wild type BRAF melanomas, suggesting these receptors are co-reactivated in melanoma to promote survival. Indeed, while blocking either pathway triggers modest effects, combining a γ-secretase inhibitor to block Notch activation, and a tyrosine kinase inhibitor to inhibit ERBB3/2 elicits synergistic effects, reducing cell viability by 90% and by hampering melanoma tumor growth. Specific inhibition of Notch1 and ERBB3 mimics these results, suggesting these are the critical factors triggering melanoma tumor expansion. Notch and ERBB inhibition blunts AKT and NFκB signaling; Constitutive expression of NFκB partially rescues cell death. Finally, blockade of both Notch and ERBB signaling inhibits the slow cycling JARID1B positive cell population, which is critical for long-term maintenance of melanoma growth. We propose that blocking these pathways is an effective approach to treat melanoma patients regardless of whether they carry mutated or wild type BRAF.

Keywords

melanoma; Notch1; ERBB3; AKT; NFκB; tumor regression

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INTRODUCTION

Small molecule therapeutics hold great promise for tumor specific targeting. In melanoma, a highly aggressive disease refractory to conventional therapies, for example, the advent of drugs specifically targeting mutated *BRAF* has extended and improved the quality of life of patients. Unfortunately, the benefits of *BRAF* inhibitors are short lived as resistance sets in within six to twelve months of treatment (Alcala and Flaherty, 2012). Furthermore, *BRAF*-targeted therapies are available exclusively to patients with mutated *BRAF*, roughly 50% of all melanoma cases. New treatments targeting alternative pathways are needed to overcome some of these challenges.

The Notch and ERBB pathways are evolutionarily conserved signaling cascades that play essential roles in embryogenesis and in cell renewal in the adult by participating in the maintenance of stem cell pluripotency in a variety of tissues (Brabletz *et al.,* 2009; Imayoshi *et al.,* 2010; Zhou *et al.,* 2010) and are required for the maintenance of melanocyte stem and precursor cell homeostasis (Birchmeier, 2009; Britsch *et al.,* 1998; Buac *et al.,* 2009; Osawa and Fisher, 2008).

While in mature melanocytes Notch1 expression is reduced, Notch1 is re-expressed and activated in melanomas, where it promotes growth, survival and progression (Bedogni *et al.,* 2008; Pinnix *et al.,* 2009; Zhang *et al.,* 2012).

The ERBB family consists of four closely related transmembrane tyrosine kinase receptors: epidermal growth factor receptor (EGFR), ERBB2 (HER2), ERBB3 (HER3) and ERBB4 (HER4). Neuregulins (NRGs) are ligands for ERBB3 and 4.

In melanoma, ERBB3 has been found activated in both tumors and cell lines (Buac *et al.,* 2009; Tworkoski *et al.,* 2011) and its expression levels associated with poor patient outcome (Reschke *et al.,* 2008); while ERBB4 activating mutations have been observed in up to 19% of melanomas (Prickett *et al.,* 2009).

Recent studies have shown a cross-talk between Notch and ERBB. For example, ERBB2 and Notch1 control their reciprocal expression in normal and cancer stem cells of the breast leading to enrichment in ERBB2/Notch expressing stem cells (Korkaya *et al.,* 2008; Magnifico *et al.,* 2009) that in the tumor favors tumorigenesis. In melanoma, we identified NRG1 as a new transcription target of Notch1 (Zhang *et al.,* 2012). NRG1 produced by Notch1 expressing cells sustains melanoma growth in part by activating ERBB3/ERBB2 signaling that triggers pro-growth/survival cues in melanoma cells (Zhang *et al.,* 2013).

Several pathways downstream of Notch1 and ERBB3/ERBB2 signaling are overlapping, suggesting that in cells where both cascades are activated they may work in concert to promote cell survival. Here we show that Notch and ERBB signaling are concomitantly activated and co-expressed in melanoma tumors regardless of whether they express mutated and wild type BRAF. The targeting of both pathways synergizes in reducing melanoma cell viability and potently hampers tumor growth. These effects are associated with blunted AKT and NFκB signaling, two major pro-survival pathways in melanoma (Amiri and Richmond, 2005; Dhawan *et al.,* 2002; Ueda and Richmond, 2006; Yang *et al.,* 2010). Finally, we

observed that the GSI/Lapatinib combination targeted subpopulations of cells expressing JARID1B, a marker for slow cycling melanoma cells that is critical for long-term maintenance of melanoma tumor growth (Roesch *et al.,* 2010).

Taken together, these data support the concomitant inhibition of Notch1 and ERBB3 as a potential effective therapy for melanoma patients.

RESULTS

Active Notch1 and active ERBB3 are co-expressed in both mutated and wild type BRAF melanomas

We have previously identified a significant association between elements in the Notch and ERBB pathways in a number of publicly available array datasets (Rhodes *et al.,* 2004) of both melanoma tissue specimens and cell lines (Zhang *et al.,* 2012) suggesting these pathways are interrelated. Here we further show that a correlation exists specifically between active Notch1 (NIC) and active (phosphorylated) ERBB3 and ERBB2 in 7 out of 9 of human melanoma samples (Fig. 1A, B), whereas no correlation was observed between $Notch1^{NIC}$ and other ERBB receptors (Fig. 1C). Also, among the ERBB receptors only the expression of p-ERBB3 and p-ERBB2 correlated significantly, further reiterating our data of the existence of an ERBB3/ERBB2 signaling unit in melanoma cells (Zhang *et al.,* 2013). Importantly, both mutated and wild type BRAF melanomas express similar levels of active Notch1 and active ERBB3 (Fig. 2A, B) and together show association between the two activated factors, confirming the previous data (Fig. 2C). Together, these data suggest Notch1 and ERBB3/ERBB2 signaling are concurrently reactivated in both mutated and wild type BRAF melanomas and may play important roles in their pathogenesis. Thus, the concomitant targeting of these pathways might have previously unappreciated therapeutic benefits.

Inhibition of ©–secretase in combination with the small molecule tyrosine kinase inhibitor lapatinib affects Notch1 and ERBB3 signaling

To address the therapeutic potential of an anti Notch-ERBB therapy, we first tested the efficacy of the GSI DBZ (dibenzazepine) and the EGFR/ERBB2 inhibitor lapatinib in inhibiting their target pathways. DBZ has a well-established activity in vivo (Milano *et al.,* 2004) and it has been extensively and successfully used in mouse leukemia models (Real *et al.,* 2009). Lapatinib is a small molecule tyrosine kinase inhibitor recently approved by the FDA for the treatment of breast cancer patients that has a similar IC50 for both EGFR and ERBB2. Lapatinb effectively hampers p-ERBB3 in melanoma cells as well as p-ERBB2 and p-EGFR (Fig. 3A). On the other hand, DBZ inhibited Notch1 processing ($Notch1^{NIC}$) and reduced HEY1 expression, a direct Notch1 target in WM266-4 cells (Fig. 3B) and in SKMel2 cells (Suppl. Fig. 1A), which are mutated *BRAF* and *RAS* driven melanoma cell lines, respectively.

Notch and ERBB signaling activate a number of pro-growth, pro-survival effectors in melanoma cells, including AKT, ERK and NFκB (Baselga and Swain, 2009; D'Altri *et al.,* 2011; Espinosa *et al.,* 2010; Koumakpayi *et al.,* 2010; Merkhofer *et al.,* 2010; Zhang *et al.,*

2006). We observed that, while ERK was not affected by either drug (Fig. 3B and Suppl. Fig. 1A), AKT phosphorylation was reduced, particularly when DBZ and lapatinib were combined (fig. 3B and Suppl. Fig. 1A). Interestingly, inhibition of Notch1 and ERBB3 by specific shRNAs led to similar results, with the higher levels of AKT inhibition when the two shRNAs were concomitantly expressed (Fig. 3C and Suppl. Fig. 1B). Additionally, NFκB activity, measured by a specific reporter construct (Bedogni *et al.*, 2008) (Fig. 3D, Suppl. Fig. 1C), was significantly inhibited in cells treated with DBZ and lapatinib, particularly when the drugs were given in combination. Anti-Notch1 and anti-ERBB3 shRNAs recapitulated the effects observed with the drugs leading to a stronger inhibition of NFκB activity when they were co-expressed (Fig. 3E, Suppl. Fig. 1D). At the molecular level, the pharmacological or genetic blockade of Notch1 and ERBB3 was associated with the retention of the NFκB subunits p65/p50 in the cytosol, likely explaining the lack of NFκB transcriptional activity (Fig. 3F, G; Suppl. Fig. 1E, F).

NFκB can be activated downstream of AKT. Blockade of Notch and ERBB inhibits AKT. Hence, to determine whether NFκB activity could be rescued by activation of AKT, we expressed a constitutive active AKT construct (Suppl. Fig 2A) in WM266-4 cells. NFκB activity was rescued but only in part, suggesting Notch and ERBB pathways converge onto NFκB both in an AKT dependent and independent manner (Suppl. Fig. 2B).

Together, these data indicate that blocking Notch and ERBB signaling by a GSI and lapatinib effectively blocks pro-survival effectors and that such effects can be recapitulated by the specific inhibition of Notch1 and ERBB3, indicating these two receptors may play key roles in the pathogenesis of melanoma.

Blockade of Notch and ERBB signaling hampers melanoma cell viability and survival

To determine whether DBZ and lapatinib could affect melanoma cells, WM266-4 cells were treated for three days with escalating doses of the drugs, either alone or in combination (Fig. 4A). Viability was then assessed by the Cell Titer-Glo assay (Promega). While lapatinib inhibited cell viability by 50% and reached a plateau at 10 μM, DBZ reduced cell viability by 20%. The effect of the two drugs together was synergistic as shown by the isobologram in Fig. 4B, and resulted in 90% loss of cell viability at 10 μM (Fig. 4A). Notably, similar effects were seen on additional cell lines, independently of whether they carried a wild type (SKMel2, S1273) or mutated BRAF (WM266-4, K457) (Fig. 4C). Combination indexes for these cell lines were calculated and indicated that these effects were synergistic at least at doses between 5–10 μM (Suppl. Fig. 3). Importantly, the drug combination induced cell death both in cells seeded in monolayer, and in cells seeded as 3D spheroids (Smalley *et al.*, 2006) (Fig. 4D, E); and induced the cleavage of PARP, indication that inhibition of Notch and ERBB signaling triggers apoptosis (Fig. 4G). The combination treatment achieved the highest levels of cell death in both assays. Notably, normal human fibroblasts were not affected by the inhibitors (Fig. 4D). These data suggest the compounds may be tumor specific with minimal toxicity to normal cells. The specific knock down of Notch1 and ERBB3 mirrored the effects of DBZ and lapatinib (Fig. 4F, H) further supporting a role of these melanocyte precursor factors in melanoma cell survival.

Inhibition of both Notch1 and ERBB3 blocks melanoma tumor growth

To further address the therapeutic potential of blocking Notch and ERBB signaling, WM266-4 cells were implanted subcutaneously in immunocompromised mice (fig. 5A). Tumors were allowed to grow to an average size of 137mm^3 in each animal, prior to treatment with DBZ (10 μmol/Kg), lapatinib (50mg/Kg) or a combination of the two given I.P. To overcome GSI related gastrointestinal side effects (Deangelo *et al.,* 2006; Searfoss *et al.,* 2003), mice were subjected to a schedule of three days on treatment followed by four days of recovery, according to recent clinical data demonstrating good GSI tolerance in patients following this regimen (Tolcher *et al.,* 2012). Indeed, the average weight of the animals did not change significantly over the experimental period (not shown), indicating the feasibility of such treatment.

Both DBZ and Lapatinib exerted cytostatic effects, whereas a combination of the two drugs reduced tumor growth rates below the initial tumor size (fig. 5A), indicating blockade of both Notch and ERBB signaling may promote tumor regression. In total, six out of eight tumors regressed in the DBZ/Lapatinib group (Suppl. Fig. 4A) and the overall decrease in tumor size between time 0 and the end time point was statistically significant (Suppl. Fig. 4B). Similar results were obtained in tumors originated from SKMel2 cells, a RAS^{Q61L} driven melanoma cell line. Although a trend in tumor regression was noted in the combination treatment, the difference between tumor sizes at time 0 and at the end time point was not statistically significant for this cell line (Suppl. Fig. 5). Nonetheless, together these data further support the potential feasibility of the treatment in both mutated and wild type *BRAF* tumors.

To determine whether the growth inhibition was specifically associated with the inhibition of Notch1 and ERBB3, cells expressing shNotch1 and shERBB3 or an shRNA control (shGFP), were implanted in immunocompromised mice (fig. 5B). Interestingly, while the growth of tumors originating from the single shRNA expressing cells was drastically delayed, tumors were never observed during the experimental period in those mice implanted with the double shRNA expressing cells indicating that the concomitant inhibition of Notch1 and ERBB3 is sufficient to block tumor formation.

These data suggest Notch and ERBB signaling are effective therapeutic targets that can be addressed by available inhibitors.

NFκB rescues cell death caused by blockade of Notch and ERBB signaling

NFκB signaling was also affected by Notch and ERBB inhibition (Fig. 3D–G; Suppl. Fig. 1E, F). NFκB is upregulated in melanoma and associated with melanoma development and dissemination (Amiri and Richmond, 2005; Dhawan *et al.,* 2002; Ueda and Richmond, 2006; Yang *et al.,* 2010). To determine if the decrease in NFκB activity accounted for the reduced cell viability and survival upon Notch and ERBB inhibition, NFκB was expressed in WM266-4 cells (Fig. 6A, B). Cells were then subjected to DBZ and lapatinib treatment and viability and cell death evaluated. The expression of p65 and p50 resulted in a 10 fold (from 2.5% to 25%) rescue in viability (fig. 6B); reduced cell death by about 50% (Fig. 6C); and

reduced PARP cleavage (Fig. 6D), in cells treated with the combined drugs, supporting a role of NFκB downstream of Notch and ERBB.

Blockade of Notch and ERBB signaling affects the JARID1B positive slow cycling cell population

The existence of a slow-cycling subpopulation of melanoma cells, marked with expression of JARID1B, a H3K4 demethylase, has been demonstrated (Roesch *et al.,* 2010). This JARID1B(+) population has been shown to be critical for long-term melanoma tumor maintenance (Roesch *et al.,* 2010). By performing FACS analysis we found that treatment with lapatinib alone led to a decrease in the number of JARID1B(−) cells and an increase by eighteen fold of JARID1B(+) cells with respect to control cells (Suppl. Fig. 6A, B). DBZ alone did not exert any effects on either population. However, DBZ reduced the population of JARID1B(+) cells in the lapatinib treated group almost to the levels of controls and DBZ alone. Given the partial rescue in survival observed with the expression of NFκB, we wanted to determine whether NFκB could also affect the JARID1B population. Interestingly, We found that activation of NFκB increased the fraction of JARID1B(+) cells only in the combination treatment (Suppl. Fig. 6C). These results suggest NFκB may play a partial role downstream of Notch and ERBB signaling in enriching the JARID1B(+) population.

In conclusion, these findings indicate that while lapatinib preferentially targets the JARID1B(−) cells, lapatinib and DBZ suppress both populations, further supporting a combined therapy to better control melanoma.

DISCUSSION

Despite significant advances in melanoma therapy, melanoma is still a deadly disease with increasing death rates each year. Part of the problem is the emergence of resistant cells within the tumor resulting in new cancers refractory to the therapy in use. Furthermore, for BRAF targeted therapies, such treatments are applicable to half the patient population. Here we demonstrate that the targeting of alternative pathways, i.e. the melanocyte precursor pathways Notch and ERBB, leads to melanoma cell death and suppresses tumor growth and because these pathways are similarly expressed by both mutated and wild type BRAF melanomas, can be directed to a larger patient population. Mechanistically, these effects are associated with the inhibition of the survival pathways AKT and NFκB, particularly when both receptors are blocked simultaneously, either by pharmacological inhibition or by specific shRNAs against Notch1 and ERBB3, suggesting these two factors are major players in melanoma cell survival. We also observed that Notch and ERBB blockade contributes to the inhibition of the JARID1B(+) population, a small fraction of slow cycling tumor cells within melanoma that has been linked to long-term tumor maintenance and propagation (Roesch *et al.,* 2010; Roesch *et al.,* 2013). Together, these data highlight alternative targets for melanoma therapy.

©–secretase inhibitors have been tested as single agents in clinical trials for their anti-cancer activity. For these agents, disease control including complete response, partial response and stable disease has been observed in patients with various types of cancer including melanoma (Tolcher *et al.,* 2012). On the other hand, lapatinib has been approved for the

treatment of breast cancer patients and a phase II clinical trial of lapatinib for the treatment of stage IV melanoma harboring ERBB4 mutations is underway (NCT01264081). However, single agent based therapies are not very effective in controlling cancer progression, mostly because of the emergence of resistant tumors. Hence, combination therapies are becoming the line of treatment. For example, the GSI MK-0752 in combination with docetaxel has demonstrated a high disease control rate or clinical benefit rate in metastatic and locally advanced breast cancer (Takebe *et al.,* 2014). Lapatinib, in combination with capecitabine, a 5-fluorouracil pro-drug, improved time to disease progression compared to capecitabine alone in patients with advanced HER2-positive breast cancer that had progressed on trastuzumab (Geyer *et al.,* 2006). Of note to melanoma, it has been shown in preclinical models that one of the mechanisms of resistance to anti BRAF therapies is the upregulation of ERBB3 (Abel *et al.,* 2013). In this instance, the combination of lapatinib with the BRAF inhibitor PLX4720 reduced tumor burden and extended latency of tumor regrowth in vivo versus PLX4720 alone (Geyer *et al.,* 2006)

In our study, we demonstrate that DBZ and lapatinib, when administered alone, achieve modest effects on cell viability and survival, whereas synergize when combined. In vivo, only with DBZ and laptinib combined we did observe partial tumor regression. This may be due to the high redundancy of Notch and ERBB pathways. Both cascades can activate the same pro-growth, pro-survival effectors such as AKT, ERK and NFκB (Baselga and Swain, 2009; D'Altri *et al.,* 2011; Espinosa *et al.,* 2010; Koumakpayi *et al.,* 2010; Merkhofer *et al.,* 2010; Zhang *et al.,* 2006). Thus, inhibiting only one pathway may still be sufficient to maintain cell growth and survival, although to a lower rate. In fact, we find that while both DBZ and lapatinib alone, or knock down of Notch1 or ERBB3 can diminish the phosphorylation of AKT and the activity of NFκB, the combination of the drugs or the blockade of both Notch1 and ERBB3 further reduce AKT and NFκB activation, indicating these pathways act in parallel to modulate such factors. Interestingly, we observed that inhibition of ERBB by Lapatinib and even more so by shERBB3, inhibited the levels of Notch1 as well. This suggests a possible feedback mechanism of stimulation of Notch by the ERBB pathway. Studies are underway in our laboratory to identify the mechanism of this potential feedback interaction.

Importantly, we demonstrate that by restoring NFκB expression, melanoma cell viability and survival is partially rescued, indicating this signaling cascade is indeed contributing to the pro-growth/ pro-survival signaling downstream of Notch and ERBB. However, given the partial rescue, these data also suggest other pathways may be involved. Further analysis is needed to determine what additional downstream cascades mediate Notch and ERBB survival in melanoma.

In addition, we found that lapatinib triggers an enrichment in the JARID1B(+) population, possibly because it preferentially targets the larger JARID1B(−) population. Addition of DBZ neutralizes in part the enrichment effect due to lapatinib. Although additional studies are needed to understand how DBZ suppresses this population of cells, e.g. by inhibiting their growth or by suppressing the switch from the JARID1B(−) to a JARID1B(+) phenotype, it appears that combining lapatinib and DBZ reduces both cell populations which can contribute to the promotion of tumor regression. Interestingly, Roesch *et al* (Roesch *et*

al., 2010) have previously shown that JARID1B^{high} melanoma cells express higher levels of the Notch targets HEY1 and HEY2, suggesting that Notch1 and JARID1B may be part of a program to regulate melanoma cell stemness. Our data may add additional complexity to the relationship between these factors suggesting a potential reciprocal regulation.

In conclusion, given that blockade of Notch and ERBB signaling effectively impairs melanoma cells regardless of whether they express wild type or mutated *BRAF*, a therapeutic approach targeting Notch and ERBB signaling can not only serve as adjuvant therapy to *BRAF* inhibitors to overcome resistance; but can extend beyond *BRAF* driven melanomas, thus providing effective therapeutic benefits to a majority of melanoma patients.

MATERIALS AND METHODS

Cells and tissue specimens

Melanoma cells used in this study were: WM266-4, K457 (mutated BRAF) and SKMEL2, S1273 (Wild Type BRAF) (a gift from Dr Marianne Broome Powell, Stanford University) (Bedogni *et al.,* 2008). Cells were maintained in DMEM (Dulbecco's modied Eagle's medium) supplemented with 10% fetal calf serum, 1% glutamine and 1% penicillin–streptomycin.

De-identified melanoma tissues were obtained from the repository of the Interdisciplinary Melanoma Cooperative Group at New York University (IRB# 10362).

Plasmids, shRNAs and Chemicals

P65 and p50 cDNAs were purchased from Addgene (pcMV4-p50, plasmid#21965 and GFP-RelA plasmid#23255, deposited by Warner Greene) and cloned into the pLM-CMV-Hapuro-PL3 lentiviral plasmid (Razorenova *et al.,* 2005). shRNAs against human Notch1 (TRCN0000003359) and human ERBB3 (TRCN0000000621) were purchased from sigma (Saint Louis, MO, USA). DBZ (dibenzazepine) was purchased from Selleck Chemicals (Houston, TX, USA); lapatinib was from LC Laboratories (Woburn, MA, USA). All compounds were dissolved in DMSO (Sigma, Saint Louis, MO, USA).

Luciferase assays

WM266-4 cells (5×10^4) were transfected with a NFκB reporter construct (Bedogni *et al.,* 2008) and a CMV-driven Renilla reporter control at a 1:20 ratio, respectively. After 36–48 h, activities of Firefly and Renilla were assessed by the dual-luciferase Assay system (Promega, Madison, WI, USA)) and light production was measured for 10 s in a Monolight 2010 Luminometer (Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis

Cells (2×10^6) were plated in complete media containing vehicle (DMSO) or compounds at the indicated doses and were collected 24 h post-treatment. Total protein was extracted with urea lysis buffer (9 M urea; 75 μM Tris–HCl, pH 7.5 and 100 μM 2-mercaptoethanol (2-ME)). Frozen tumor samples were homogenized in RIPA buffer (50 μM Tris–Cl, pH 7.4; 150 μM NaCl; 1% NP-40; 0.25% Na-deoxycholate; 1 μM PMSF; 1 μg/ml aprotinin; 1 μg/ml

leupeptin; and 1 μM Na-ortovanadate). Nuclear/cytosolic fractions were prepared by disrupting in a Dounce homogenizer in a hypotonic buffer (20 μM HEPES, pH 7; 10 μM KCl; 1 μM MgCl2; 0.1% Triton X-100; 20% glycerol; 2 μM PMSF; 5 μg/ml aprotinin; and 5 μg/ml leupeptin) and centrifuged at 850 g to obtain a pellet of nuclei. 40–50 μg per sample (either cell, nuclear or tumor lysates) were separated by 8–10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Antibodies against phosphorylated and total ERBB receptors, phosphorylated and total AKT and ERK, p65, p50, Notch1 and cleaved PARP are from Cell Signaling Technologies (Beverly, MA, USA). Loading was normalized with anti β-actin, GAPDH or α-tubulin for total and cytosolic lysates, respectively, and Lamin-B1 and HDAC1 for nuclear lysates (Santa Cruz Biotechnology).

Viability and cell death assay

Viability was evaluated by the Cell titer-Glo (Promega, Madison, WI, USA) as per manufacturer's instructions on 3000 cells seeded in triplicate in 96-well plates and treated for three days with 10 μM DBZ and lapatinib. Cell death was calculated on cells treated as above by using trypan blue.

Melanoma spheroids

200 μL of K457 cells (25,000/mL) were added to a 96-well plate coated with 1.5% agar (Difco, Sparks, MD). Cells were incubated for three days to allow formation of spheroids. Spheroids were harvested and implanted into collagen I/complete media gels containing the drugs. After three days incubation, spheroids were washed in PBS and stained with calcein-AM and ethidium bromide (Molecular Probes, Eugene, OR) for 1 hour according to the manufacturer's instructions. Pictures were taken using a Nikon-300 inverted fluorescence microscope.

In vivo tumor growth

Cells (2×10^6) were injected subcutaneously in the dorsal flanks of 8 weeks old Male SCID mice for a total of eight tumors per experimental group. Mice were supplied by the Athymic Animal Facility at Case Western Reserve University under an Administrative Panel on Laboratory Animal Care approved protocol. Tumors were measured with a caliper and tumor volumes calculated.

Flow Cytometric Analysis

For JARID1B expression, we followed procedures published in Yuan et al (Yuan *et al.*, 2013). Briefly, cells were fixed in 1% formalin in PBS for 20 min, followed by incubation with 90% methanol for 30 min at −20 °C. After washing with 1% FBS in PBS, anti-JARID1B antibody (Novus Biologicals, Littleton, CO), was added to the cells and incubated for 30 min at room temperature. After washing, the cells were stained with Alexa Fluor 647 conjugated anti-rabbit IgG. The samples were analyzed by BD LSRII Flow Cytometer. All flow cytometry data were analyzed with FlowJo software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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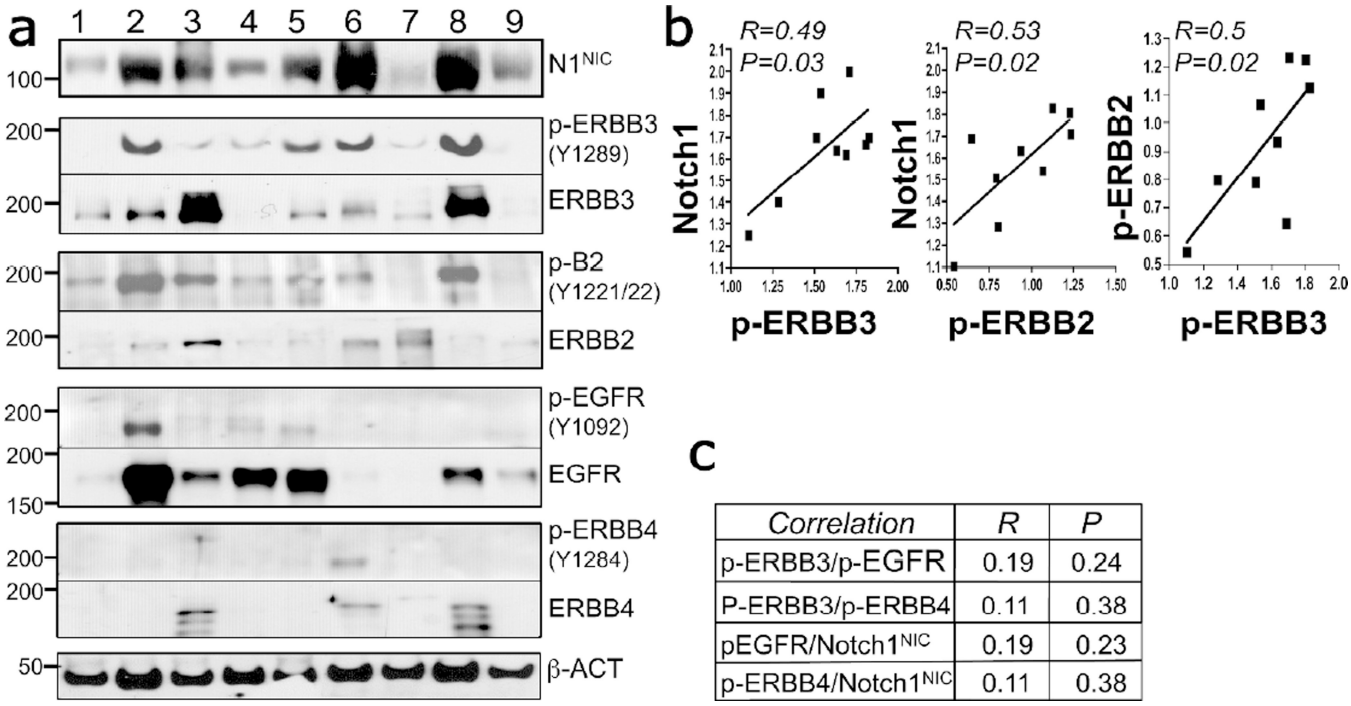

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Correlation	R	P
p-ERBB3/p-EGFR	0.19	0.24
P-ERBB3/p-ERBB4	0.11	0.38
pEGFR/Notch1NIC	0.19	0.23
p-ERBB4/Notch1NIC	0.11	0.38

Figure 1. Notch1^{NIC} and phosphorilated ERBB3/ERBB2 correlate in melanoma

A) Expression levels of phosphorylated and total ERBB receptors and of active Notch1 (Notch1^{NIC}) in nine flash-frozen human metastatic melanomas. ***B)*** Correlation analysis between Notch1^{NIC} and phospho-ERBB3, phospho-ERBB2 and between phospho-ERBB3 and 2. ***C)*** Lack of correlation between the other components analyzed in A.

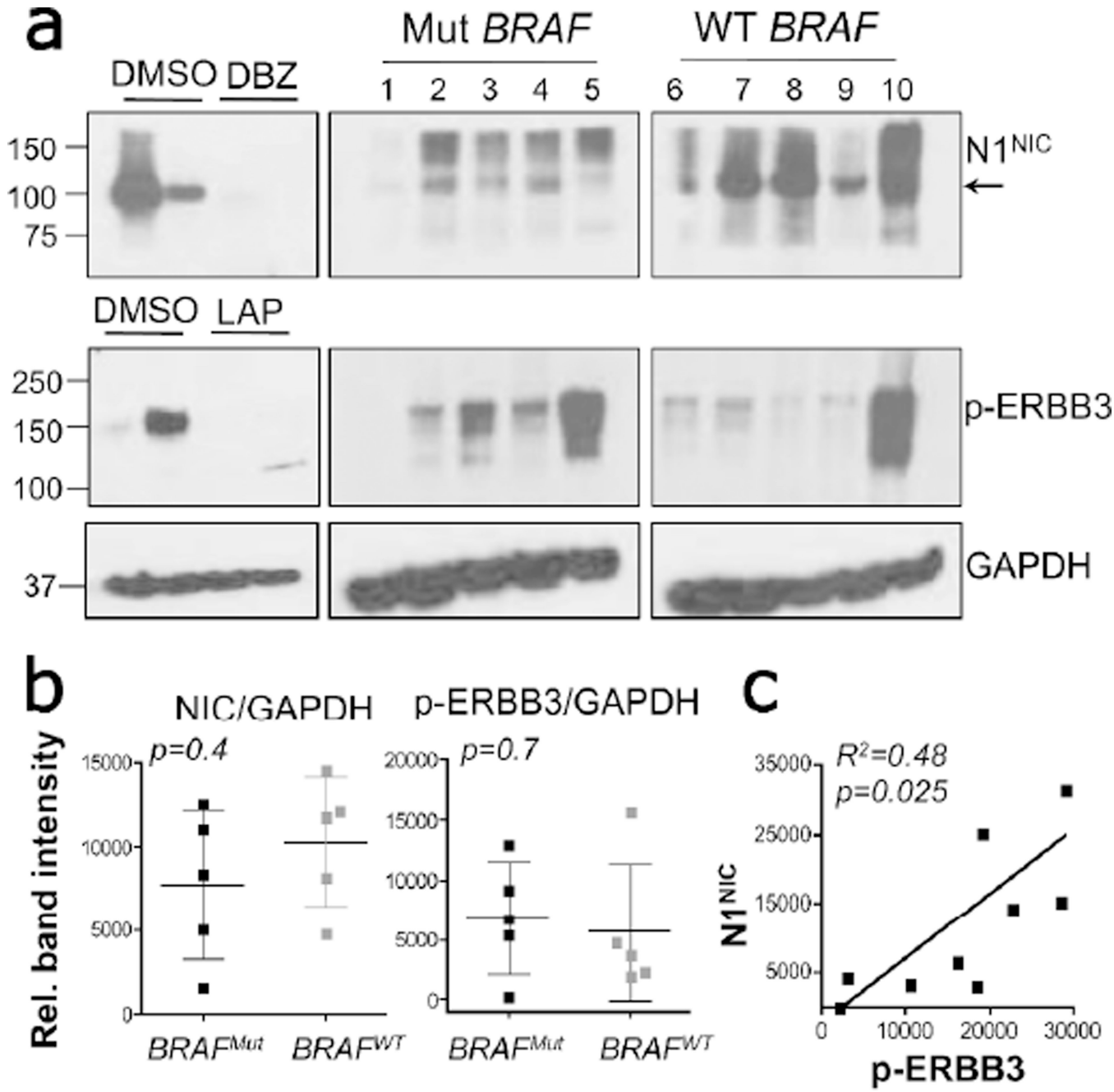

Figure 2. Notch1^{NIC} and poshpo-ERBB3 are similarly expressed in mutated and wild type BRAF melanoma tumors

A) Expression levels of Notch1^{NIC} (upper panel) and phoshporylated ERBB3 (lower panel) in five mutated and five wild type BRAF melanoma tumors. Cells treated with DBZ (10 μM) and lapatinib (10 μM) O/N (left panels) are used as positive controls for Notch1^{NIC} and phopho-ERBB3, respectively. GAPDH is used a loading control. **B)** Quantification of the bands in A normalized to their correspondent loading control. No statistical difference in expression of either Notch1^{NIC} or phospho-ERBB3 between mutated and wild type tumors

was observed (student's T test). **C)** Correlation between Notch1^{NIC} and phospho-ERBB3 among all samples in A.

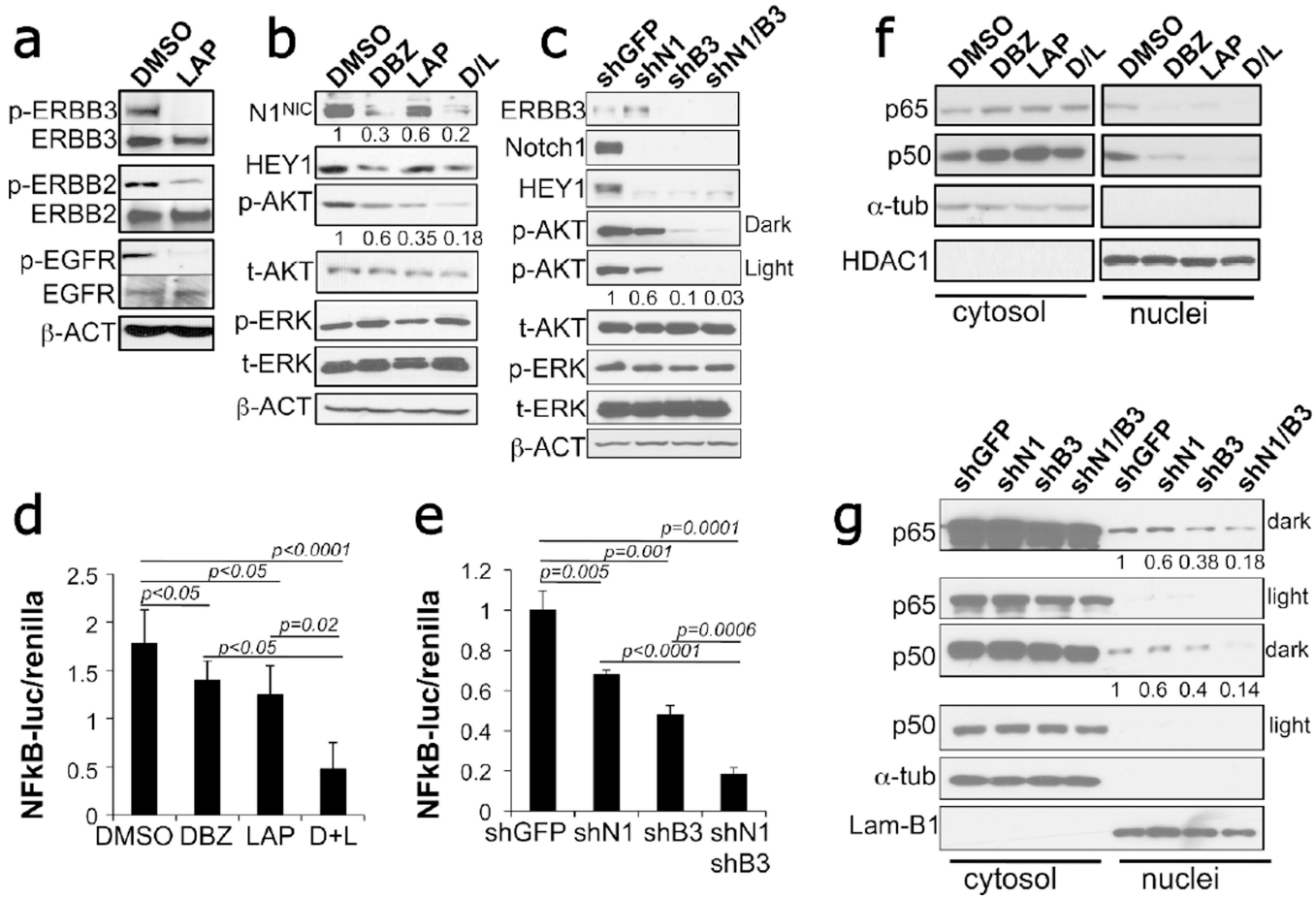

Figure 3. Inhibition of Notch and ERBB inhibits AKT and NFκB activity

A) Effects of lapatinib (10μM) on the phosphorylation levels of ERBB3, ERBB2, EGFR in WM266-4 cells. ***B)*** DBZ (10μM) and lapatinib (10μM) effects on Notch1^{NIC}, HEY1, phosphorylated AKT and ERK1/2 in WM266-4 cells. Numbers represent quantification of band intensity normalized to their respective loading control and are the average between three independent western blots. ***C)*** Expression levels of Notch1, ERBB3, HEY1, phosphorilated AKT and ERK in cells (WM266-4) expressing specific shRNAs against Notch1 and ERBB3. Numbers represent quantification of band intensity normalized to their respective loading control and are the average between three independent western blots. ***D–E)*** NFκB Reporter assay in cells treated with DBZ and lapatinib or expressing shGFP, shNotch1, shERBB3 or the two together. P values are calculated by the Student's T test. Data are the average±SD between at least three independent experiments. ***F–G)*** Cytosolic and nuclear distribution of the NFκB elements p65 and p50 in DBZ/lapatinib treated cells (**F**) or in cells expressing shRNAs against Notch1 and ERBB3 (***G***).

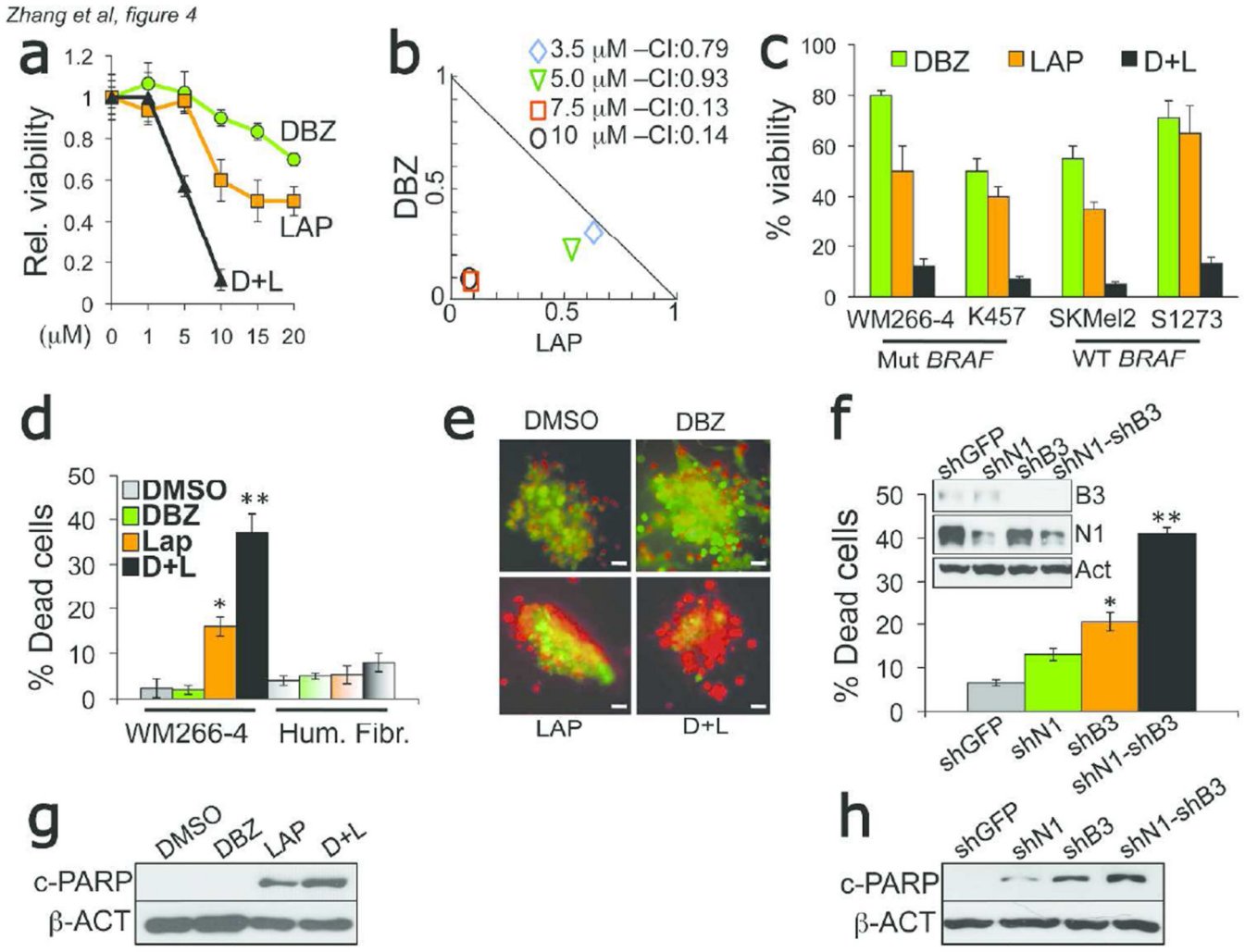

Figure 4. Blockade of Notch and ERBB hampers melanoma cell viability

A) Viability at three days of WM266-4 cells treated with escalating doses of DBZ and lapatinib. ***B)*** Isobologram indicating synergism between DBZ and lapatinib at concentrations 5–10 μM (calculated using the free software CompuSyn ([Chou and Martin, 2007]). ***C)*** Viability at three days of BRAF mutated (WM266-4, K457) and wild type (SKMEL2, S1273) cells in response to 10 μM DBZ and lalatinib. ***D)*** Cell death evaluated by trypan blue exclusion of WM266-4 cells or human fibroblasts treated for three days with DBZ and lapatinib. *,**p<0.001. Student's t test. ***E)*** K457 spheroids treated for three days with DBZ and lapatinib, stained with Calcein AM (viable cells- green) and Ethidium Bromide (dead cells- red). ***F)*** Cell death evaluated by trypan blue exclusion of WM266-4 expressing shNotch1 and shERBB3, alone or combined. *,**p<0.001, Student's t test. ***G–H)*** PARP cleavage in WM266-4 cells treated with the compounds ***(G)*** or expressing shNotch1 and shERBB3 ***(H)***.

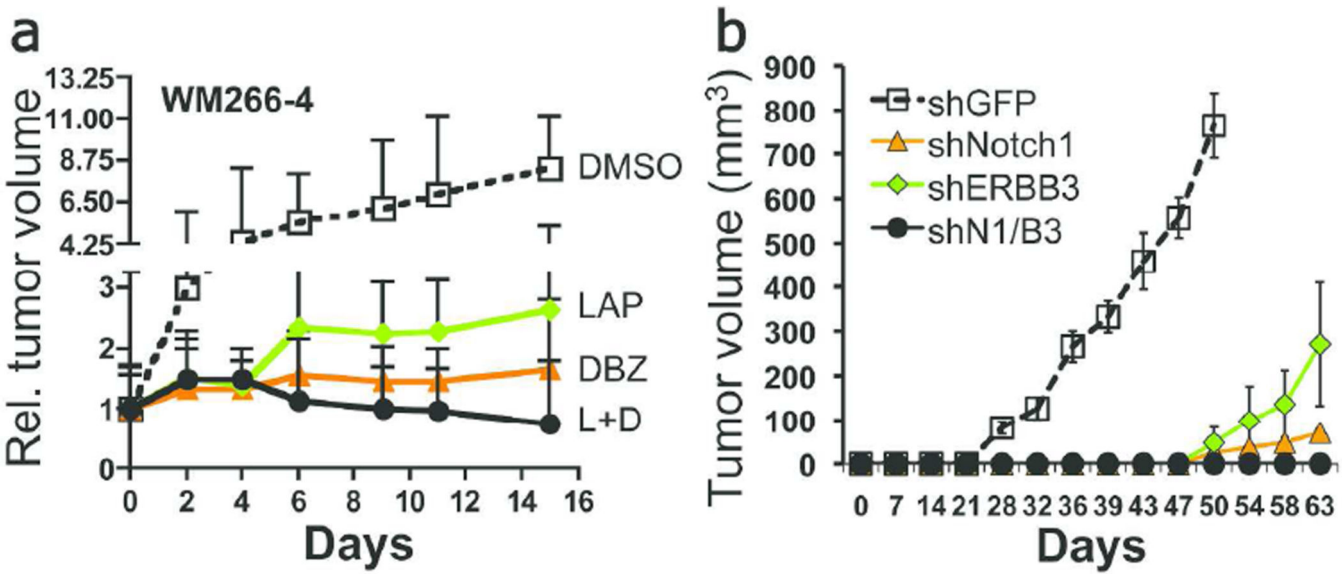

Figure 5. Inhibition of Notch1 and ERBB3 blocks tumor formation

A) Growth of WM266-4 cells in SCID mice (n=8). Tumor volumes were normalized to 1 at time 0 (mean vol. 137 μM^3), time at which mice were given 50 mg/Kg lapatinib and 10 μMol/Kg DBZ thrice a week. (LAP vs DMSO, *p=0.007*; DBZ vs DMSO, *p=0.002*; DBZ +LAP vs DMSO, p=0.001; DBZ+LAP vs LAP, *p=0.008*; DBZ+LAP vs DBZ, *p=0.05*, Student's t test). ***B)*** Growth of WM266-4 cells expressing shRNA control (shGFP) or shRNAs against Notch1 and ERBB3, in SCID mice (n=10). (shGFP vs shNotch1, p<0.001; shGFP vs shERBB3, p<0.001; shGFP vs shN1/shB3, p<0.001; shN1/B3 vs shNotch1, p=0.002; shN1/B3 vs shERBB3, p=0.001, Student's t test).

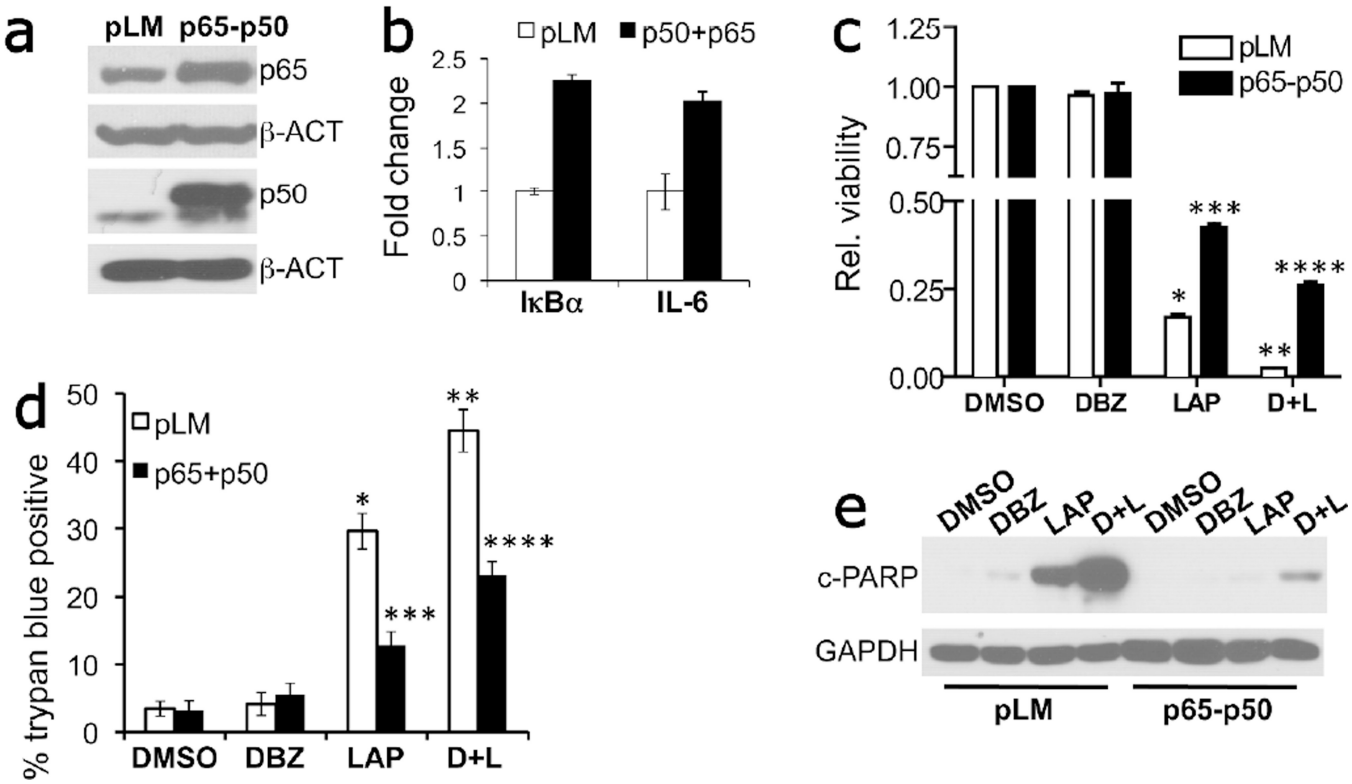

Figure 6. Restoration of NFκB rescues viability and death due to Notch and ERBB inhibition

A) Expression of p65 and p50 NFκB elements in WM266-4 melanoma cells. β-actin was used as loading control. ***B)*** qRT-PCR for the NFκB targets IκBα and IL-6. Values were normalized to GAPDH and fold change over pLM (set at 1) is indicated. ***C)*** Viability of the cells in A treated for three days with 10 μM DBZ and lapatinib. *$DMSO_{pLM}$ vs LAP_{pLM}, p<0.0001; **$DMSO_{pLM}$ vs $D+L_{pLM}$, p<0.0001; ***LAPpLM vs LAPp65-p50, p<0.0001; ****D+LpLM vs D+Lp65-p50, p<0.0001). ***D)*** cell death by Trypan blue exclusion assay in cells expressing pLM (empty vector) or p65-p50 and treated with the compounds for two days. *$DMSO_{pLM}$ vs LAP_{pLM}, p<0.0001; **$DMSO_{pLM}$ vs $D+L_{pLM}$, p<0.0001; ***LAPpLM vs LAPp65-p50, p<0.0001; ****D+LpLM vs D+Lp65-p50, p<0.0001). ***E)*** cleaved PARP of the cells in C.