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Monoclonal antibody-induced ErbB3 receptor internalization and degradation inhibits growth and migration of human melanoma cells

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Members of the ErbB receptor family are targets of a growing numbers of small molecules and monoclonal antibodies inhibitors currently under development for the treatment of cancer. Although historical efforts have been directed against ErbB1 (EGFR) and ErbB2 (HER2/neu), emerging evidences have pointed to ErbB3 as a key node in the activation of proliferation/survival pathways from the ErbB receptor family and have fueled enthusiasm toward the clinical development of anti-ErbB3 agents. In this study, we have evaluated the potential therapeutic efficacy of a set of three recently generated anti-human ErbB3 monoclonals, A2, A3 and A4, in human primary melanoma cells. We show that in melanoma cells expressing ErbB1, ErbB3 and ErbB4 but not ErbB2 receptor ligands activate the PI3K/AKT pathway, and this leads to increased cell proliferation and migration. While antibodies A3 and A4 are able to potently inhibit ligand-induced signaling, proliferation and migration, antibody A2 is unable to exert this effect. In attempt to understand the mechanism of action and the basis of this different behavior, we demonstrate, through a series of combined approaches, that antibody efficacy strongly correlates with antibody-induced receptor internalization, degradation and inhibition of receptor recycling to the cell surface. Finally, fine epitope mapping studies through a peptide array show that inhibiting vs. non-inhibiting antibodies have a dramatically different mode of binding to the receptor extracellular domain. Our study confirms the key role of ErbB3 and points to exploitation of novel combination therapies for treatment of malignant melanoma.

Introduction

Malignant melanoma is the deadliest form of skin cancer for its high proliferative rate and propensity to establish metastasis in several organs.[1] Although treatment options have recently increased thanks to the approval of two new drugs with distinct mechanisms of action, namely, immunotherapy with the monoclonal anti-CTLA4 antibody ipilimumab[2] and targeted therapy with vemurafenib,[3] a selective BRAF inhibitor for patients carrying the V600E BRAF mutation, the 5-year survival still remains very low due to the intrinsically highly aggressive and metastatic nature of this type of cancer as well as, in the case of BRAF inhibitors, to the rapid development of resistance.[4] Hence, research is constantly pursuing additional strategies for intervention that could allow the development of more effective combination treatments.

During the last two decades, members of the EGF receptor family have become prominent targets for the treatment of several types of cancers thanks to their key role in the activation of the PI3K/Akt proliferation and survival signaling pathways.[5,6] The EGFR superfamily of receptor tyrosine kinases consists of four distinct members, namely, ErbB1 (EGFR/HER1), ErbB2 (HER2/neu), ErbB3 (HER3), ErbB4 (HER4), which are spatially and temporally regulated by a wealth of ligands.[7] Small molecules and monoclonal antibodies developed against two members of this family, namely, EGFR and HER2, have demonstrated therapeutic efficacy and have been approved for the treatment of several forms of cancers, including breast, lung, colorectal, gastric and head and neck cancers.[7]

In recent years, ErbB3 has emerged as a key player in the establishment of malignancy.[8] Disregarded for several years, largely because of the absence of detectable ErbB3 mutations in cancer samples and for the lack of a functional kinase domain in its intra-cytoplasmic region,[9] ErbB3 is instead one of the most potent activators of the PI3K/Akt axis because of the presence of six tyrosine residues in its intracytoplasmic tail, many

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of which, when trans-phosphorylated by other receptor tyrosine kinases (RTKs), become high affinity binding sites for the regulatory subunit of PI3K.[10] ErbB3 has been shown to be an obligate partner for ErbB2-mediated transformation[11,12] and is upregulated and trans-phosphorylated in several forms of cancer, in particular following treatment with EGFR and ErbB2 inhibitors.[13,14] Hence, a growing number of evidences point to ErbB3 as an important factor in the establishment of resistance to therapies. Furthermore, ErbB3 overexpression and its activation of the PI3K pathway have been shown to be required for the early metastatic steps of motility and invasion by cancer cells.[15]

The relevance of EGFR family members in melanoma has been the subject of a limited number studies, and this in spite of the evidence of high-level expression during malignant progression[16] and the importance of the activation of the PI3K/Akt pathway in progression to metastasis as well in this form of cancer.[17] More recently, it has been suggested that ErbB3 signaling is capable of inhibiting differentiation genes in melanocytes and melanoma cells and of promoting their proliferation.[18] This led us to postulate that targeting ErbB3 activation may provide a new therapeutic option for malignant melanoma.

Due to the lack of intrinsic tyrosine kinase activity, ErbB3 cannot be targeted by canonical TKIs. However, its inhibition can be achieved by monoclonal antibodies recognizing the extracellular domain. Indeed, monoclonal antibodies against human ErbB3 have been generated in recent years and have been shown to inhibit proliferation in vitro and in vivo of several cancer cell lines.[19-21] Our group has recently generated and characterized a set of mouse monoclonal antibodies against human ErbB3 obtained by immunizations with a mutant form of the receptor, which constitutively adopts and extended conformation.[22] We showed that our antibodies are able to bind human ErbB3 with low nM affinity, inhibit heregulin-induced signaling and affect cell proliferation in vitro and in vivo in a variety of cell lines. Most interestingly, one of our anti-ErbB3 monoclonal antibodies, which cross-reacts with the murine ErbB3, was able to dampen growth of ErbB2-dependent spontaneous tumors in BALB/NeuT transgenic mice.

In the present study, we have evaluated the effect of anti-human ErbB3 monoclonals on melanoma cell growth and migration. We provide evidence that anti-ErbB3 monoclonals with similar receptor affinities but with distinct ability to induce receptor internalization and degradation, exert dramatically different effects on inhibition of cell signaling, proliferation and migration. Furthermore, in order to provide further insights into their mechanism of action, we compare the intracellular fate of ErbB3 receptor after cell exposure to inhibiting antibodies with that induced by heregulin and establish correlations between antibody efficacy and receptor internalization/degradation. Finally, epitope mapping studies provide the evidence that internalizing vs. non-internalizing antibodies interact with different domains of the receptor extracellular region. Our results indicate that antibodies capable of inducing ErbB3 internalization and degradation have the features required of potential therapeutic agents for melanoma.

Results

A family of anti-ErbB3 mAbs differentially interfere with receptor signaling in melanoma cells. In order to study the effect of anti-ErbB3 monoclonal antibodies, we performed an initial screening of primary melanoma cell lines (not shown) for the expression of members of the ErbB receptor family. This led to the identification of two cell lines, MST-L and Mel 501, which express high levels of EGFR and ErbB3 but not of ErbB2, the most potent ErbB3 partner in cell transformation.[12] We decided to focus our attention on these two cell lines as representative example of melanomas, where ErbB3 could exert a prominent role in the activation of PI3K/Akt signaling, proliferation and migration.

To assess the level of expression of members of ErbB family in these melanoma cell lines, western blot analysis were performed using extracts of cells grown in the presence of FBS or serum starved and then treated with HRG. Results (**Fig. 1A**) showed a band corresponding to the molecular weight of ErbB3 both in Mel 501 and MST-L cells. Moreover, in both cell lines, specific bands corresponding to the molecular weight of ErbB1 and ErbB4 were also detectable, while no band corresponding to ErbB2 protein was visible. HeLa cells, expressing high levels of ErbB1, ErbB2 and ErbB4 but no detectable ErbB3 were used as control.

We then examined whether the ErbB3 receptors would be correctly exposed on the cell surface and if their amount would be sufficient to be visualized by immunostaining. Immunofluorescence analysis was performed by incubating unstimulated living cells at 4°C with a commercial anti-ErbB3 antibody directed against the extracellular portion of the receptor. Nuclei were labeled with DAPI. Receptor staining was clearly detectable at the level of the plasma membrane and appeared uniform in both Mel 501 and MST-L (**Fig. 1B**). In contrast, HeLa cells were unlabeled, as expected (**Fig. 1B**).

Since ErbB3 is the most potent activator of the PI3K/Akt axis, and many evidences point to the ligand-dependent activation of PI3K/Akt signaling through heterodimerization of ErbB3 with other ErbBs or RTKs as crucial events in the establishment of resistance to anti-EGFR and anti-ErbB2 therapies in many different cancers,[8] we analyzed PI3K/Akt signaling in Mel 501 and MST-L cells and tested their responsiveness to exogenous HRG. Cells were grown in the presence of FBS or serum starved and then stimulated with HRG. The amount of ErbB3 and Akt phosphorylation was assessed by western blot analysis using anti-pErbB3 and anti-pAkt antibodies. As shown in **Figure 2A**, ErbB3 phosphorylation was evident only upon HRG treatment in both cell types, while basal Akt phosphorylation was clearly increased after ligand stimulation. Thus, the PI3K/Akt signaling can be highly induced by HRG stimulation in both melanoma cells and is probably triggered by ErbB3 heterodimerization with ErbB1 and/or ErbB4.

Three distinct anti-ErbB3 monoclonal antibodies (mAbs) named A2, A3 and A4 having similar binding affinity for the receptor (K_d 5.5 nM ± 0.66; 4.40 nM ± 0.31; 2.43 nM ± 0.13 for A2, A3 and A4, respectively) but different capability to inhibit

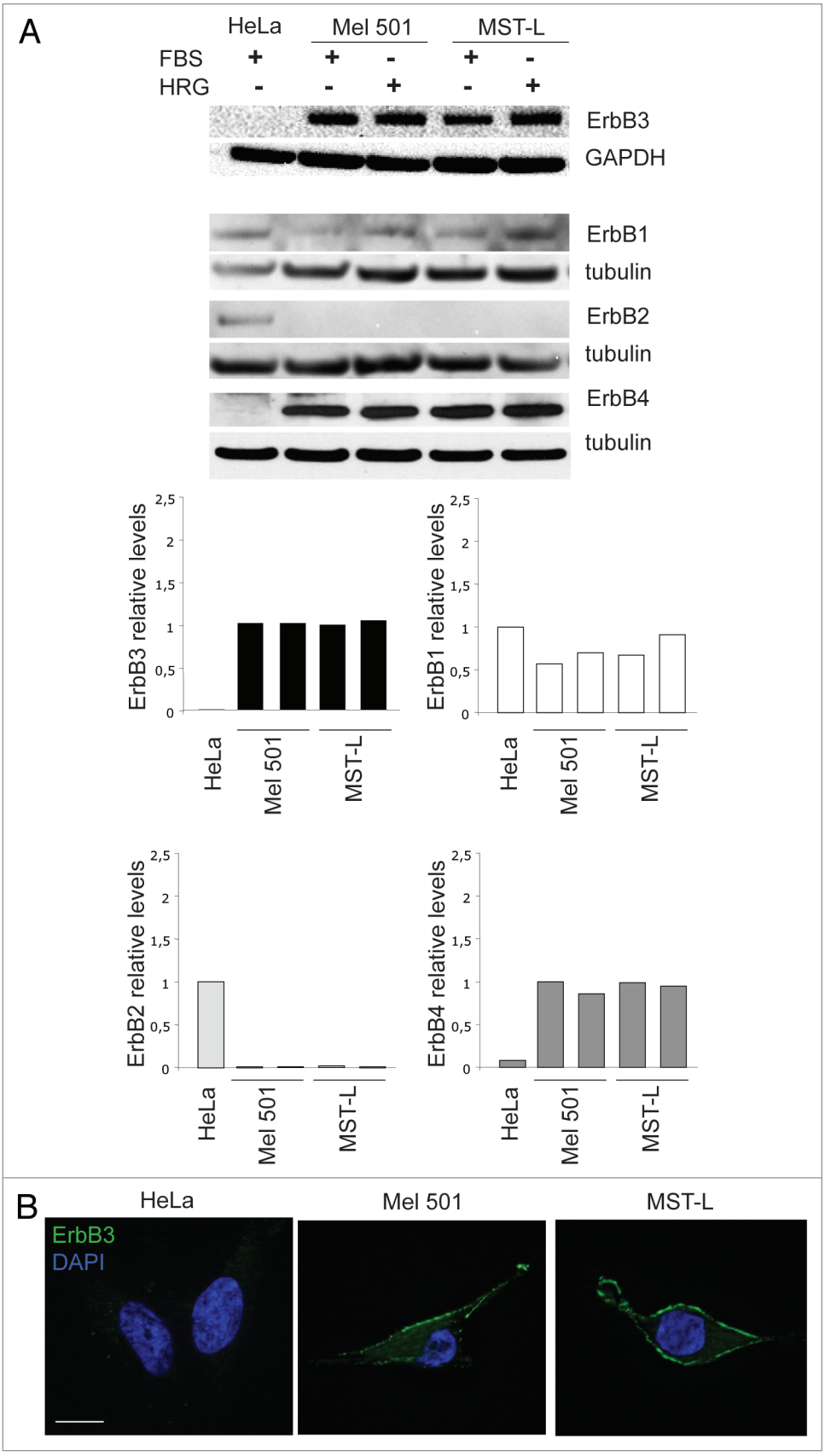

Figure 1. (A) Western blot analysis performed using specific anti-ERBB3, anti-ERBB1, anti-ERBB2 and anti-ERBB4 antibodies in melanoma cells grown in presence of FBS or serum starved and then treated with HRG for 10 min at 37°C. Specific bands at the molecular weight corresponding to the ERBB3, ERBB1 and ERBB4 proteins are visible in Mel 501 and MST-L cells, while no bands corresponding to ERBB2 protein are detected. Control HeLa cells express ERBB1, ERBB2 and ERBB4, but not ERBB3 proteins. The equal loading was assessed with anti-tubulin or anti-GAPDH antibodies and densitometric analysis was performed as described in materials and methods. (B) Immunofluorescence analysis incubating living cells at 4°C with an anti-ERBB3 antibody directed against the extracellular portion of the receptor. Nuclei were labeled with DAPI. Receptor staining is evident and uniform on the plasma membrane of both Mel 501 and MST-L; HeLa cells appear unlabeled. Bar = 10 μm.

heregulin ligand binding [72.8 nM ± 42.3; 1.0 nM ± 0.4; 2.7 nM ± 0.4 for A2, A3 and A4, respectively (**Table S1**)] were previously generated by our group.[22] In order to assess whether they could interfere with the HRG-dependent Akt signaling in melanoma cells, Mel 501 and MST-L were serum starved, pre-incubated with each anti-ErbB3 mAb for 1 h at 37°C and then stimulated with HRG for 10 min in the presence of the antibodies. Western blot analysis showed that the bands corresponding to pErbB3 and pAkt decreased following incubation with saturating amounts of A3 and A4 mAbs (**Fig. 2B**). Since A3 and A4 mAbs specifically bind ErbB3,[22] these results indicate that the ligand-dependent activation of the Akt pathway is mainly mediated by ErbB3 in both Mel 501 and MST-L cells. In contrast, pre-incubation with the A2 mAb produced no effect on HRG-induced receptor and Akt phosphorylation (**Fig. 2B**).

Anti-ErbB3 mAbs differentially affect melanoma cell growth and migration. In order to provide further insights into the biological effect of the three anti-ErbB3

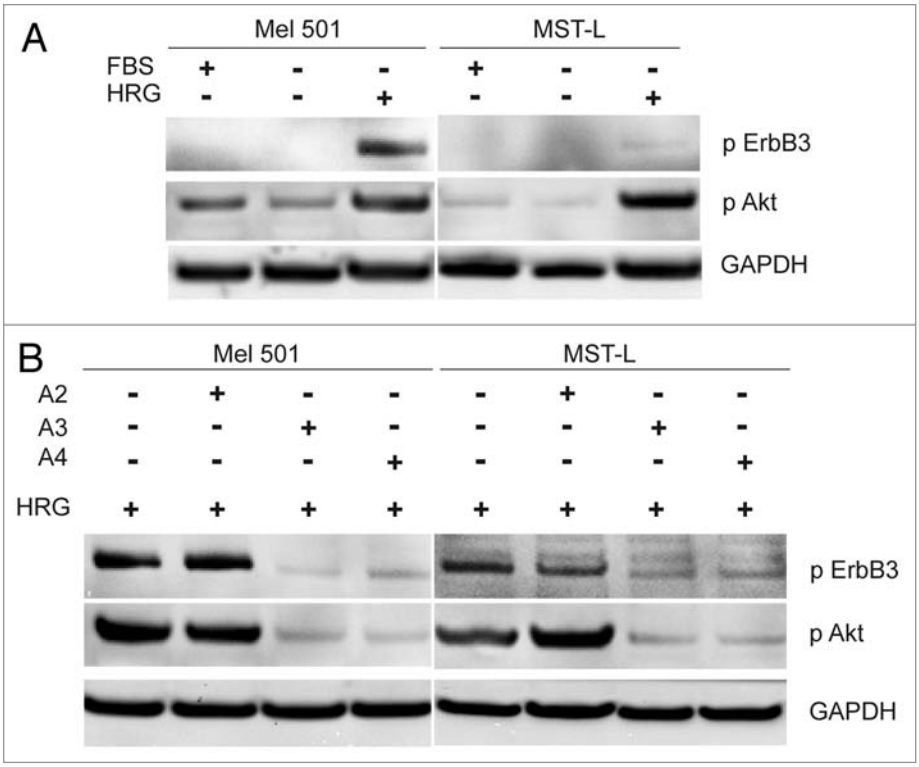

Figure 2. (A) Western blot analysis performed using anti-pERBB3 and anti-pAkt antibodies in Mel 501 and MST-L cells grown in the presence of FBS or serum starved and then stimulated with HRG for 10 min as reported in materials and methods. ERBB3 phosphorylation is evident only upon HRG treatment in both cell types. The basal Akt phosphorylation is visible in FBS-treated or serum-starved cells and is increased by ligand stimulation. The equal loading was assessed with anti-GAPDH antibodies. (B) Western blot analysis in Mel 501 and MST-L cells serum starved pre-incubated with each anti-ERBB3 mAb for 1 h at 37°C and then stimulated with HRG as above in the presence of the antibody. The phosphorylation of ERBB3 and Akt induced by HRG treatment is greatly decreased following incubation with A3 and A4 mAbs; pre-incubation with the A2 mAb does not affect HRG-induced phosphorylation of either receptor or Akt. The equal loading was assessed with anti-GAPDH antibodies.

monoclonals, we compared the proliferative response of Mel 501 and MST-L cells pre-treated with each mAb and then incubated with HRG for 48 and 72 h in the presence of the mAbs. After incubation, cells were fixed and stained with anti-Ki67 antibodies to identify cycling cells. Quantitative analysis of the percentage of cells presenting Ki67-positive nuclei indicated that the increase of the proliferation rate, clearly evident upon 48 and 72 of HRG stimulation, was strongly and significantly inhibited only by A3 and A4 mAbs (**Fig. 3**). In contrast, the minor decrease of Ki67-positive cells induced by the A2 mAb was not significant (**Fig. 3**).

Since it has been reported that some anti-EGFR mAbs are able to impede both proliferation and migration of some tumor cell lines in which the receptor is overexpressed,[23] we wondered whether, A2, A3 and A4 mAbs could also differently affect migration. Since no significant differences emerged between Mel 501 and MST-L cells in the proliferative assay, we decided to perform a migration assay only on MST-L cells. Results (**Fig. 4**) clearly indicated that HRG stimulated cell migration, and that A3 and A4 mAbs strongly interfere with the migrating effect induced by the ligand of ErbBs (**Fig. 4**), while the A2 mAb did not affect the response to HRG (**Fig. 4**). In conclusion, A3 and A4, but not A2, are able to significantly interfere with the biological activity of HRG by blocking melanoma cell proliferation and migration.

Anti-ErbB3 mAbs differentially affect receptor endocytosis and degradation. In order to provide insights into the mechanism of action of our antibodies, we decided to study the fate of ErbB3 receptor upon antibody ligation. It has been reported that anti-ErbBs mAbs can inhibit the downstream signaling inducing receptor downregulation. This has been related to antibody-induced receptor internalization and consequent degradation.[24,25] Therefore, we wondered whether the different interfering effect of A2, A3 and A4 on ErbB3 phosphorylation and signaling can be linked to a different ability to trigger ErbB3 internalization.

Mel 501 and MST-L cells were treated with A2, A3 or A4 mAbs for 1 h at 37°C before fixation to allow endocytosis of antibody receptor complexes. Immunofluorescence analysis performed using a secondary anti-mouse FITC antibody clearly showed that in both cell lines, the signal corresponding to either A3 or A4 antibodies appeared clustered in endocytic, intracellular dots dispersed throughout the cytoplasm (**Fig. 5A**); in contrast, the signal of A2 antibody remained uniformly distributed on the plasma membrane (**Fig. 5A**). Based on the observation that A2 antibody alone is not able to trigger ErbB3 endocytosis and does not compete with the ligand for binding to the receptor, we incubated melanoma cells simultaneously with HRG and A2 antibody: the signal of A2 mAb bound to ErbB3 became intracellular as a consequence of ligand-dependent internalization (**Fig. 5A**).

To quantify the amount of ErbB3 receptors that remained localized on the plasma membrane upon incubation with the three mAbs, internalization experiments were performed, incubating Mel 501 and MST-L cells with each of the mAbs for 1 h at 4°C in order to permit binding on the plasma membrane only (**Fig. 5B**, upper parts) or for 1 h at 37°C as above to allow the possible uptake of the receptor-antibody complexes (**Fig. 5B**, lower parts). The plasma membranes were visualized by incubating the cells with the lipophilic tracer Vybrant DiI at 4°C before fixation. Quantitative immunofluorescence analysis showed that, upon incubation at 4°C, the signals corresponding to all three mAbs highly colocalized with DiI (ranging from 80% to 95%) at the cell plasma membranes, indicating efficient binding in conditions of internalization block. In contrast, upon incubation at 37°C, the colocalization of A3 and A4 with DiI, but not that

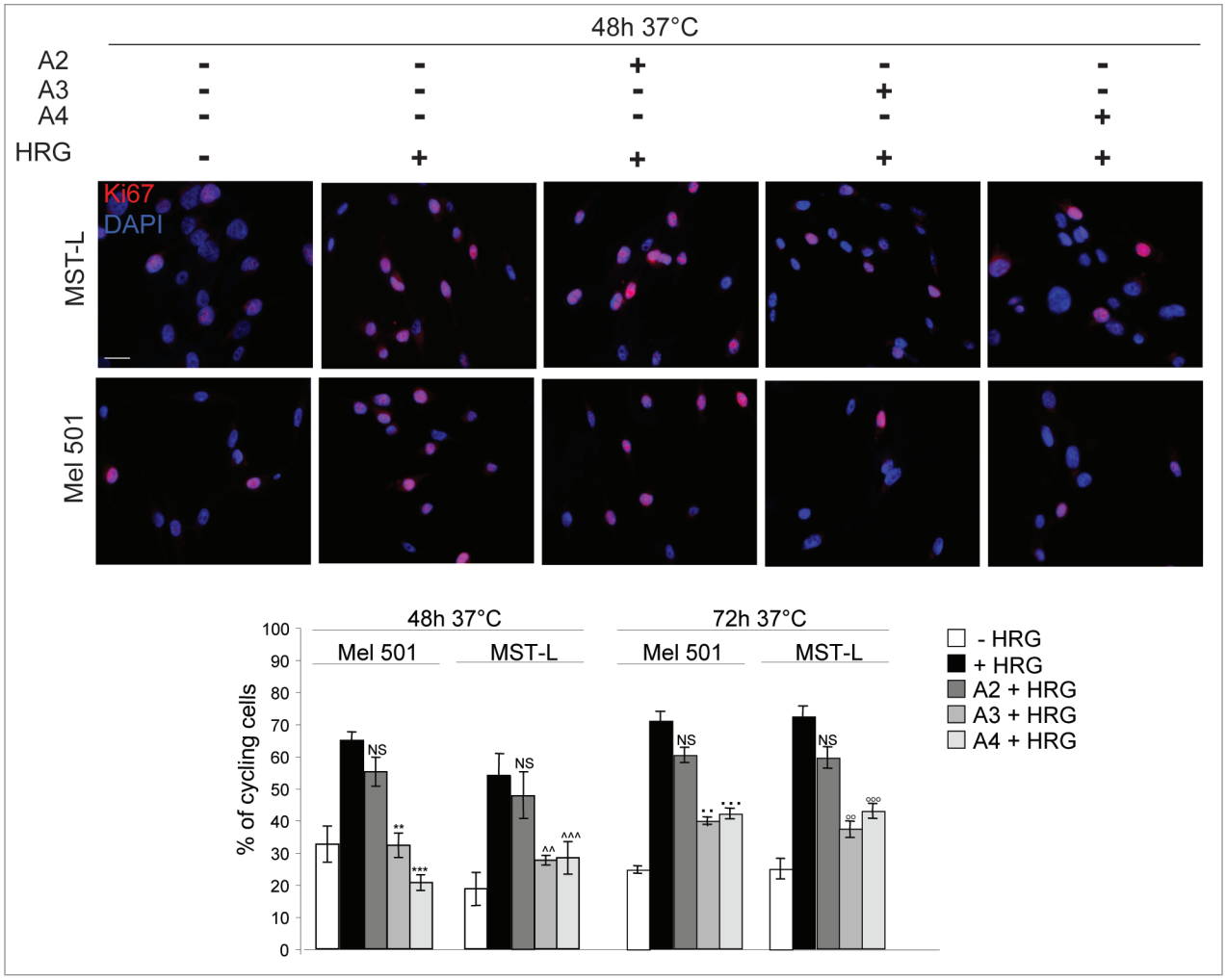

Figure 3. Mel 501 and MST-L cells were pre-treated with each mAb as above and then incubated with HRG for 48 and 72 h in the presence of the antibodies, fixed and stained with anti-Ki67 antibodies to identify cycling cells. Quantitative analysis of the percentage of cells presenting Ki67-positive nuclei was performed as reported in materials and methods and values are expressed as mean values ± standard errors (SE). Student's t test was performed and significance level has been defined as described in materials and methods. The increase of the proliferation rate upon 48 and 72 h of HRG stimulation is inhibited by the presence of A3 and A4 mAbs; the slight decrease of Ki67-positive cells induced by the A2 mAb is not significant. **p < 0.0001 vs. the corresponding HRG-treated cells, ***p < 0.0001 vs. the corresponding HRG-treated cells, ^^p < 0.001 vs. the corresponding HRG-treated cells, ^^^p < 0.001 vs. the corresponding HRG-treated cells, ¨p < 0.001 vs. the corresponding HRG-treated cells, ¨¨p < 0.001 vs. the corresponding HRG-treated cells, °p < 0.0001 vs. the corresponding HRG-treated cells, °°°p < 0.0001 vs. the corresponding HRG-treated cells. Bar = 10 μm.

of A2, was significantly reduced (**Fig. 5B**). The slight decrease in the percentage of colocalization of A2 mAb with the plasma membrane marker at 37°C compared with the binding at 4°C was not significant.

It has been previously shown in tumor cells in which ErbB3 is highly expressed that this receptor does not undergo efficient ligand-dependent downregulation.[26] In order to clarify whether the internalization of ErbB3 caused by A3 and A4 antibodies, differently from that triggered by the ligand HRG, might induce receptor targeting to the endocytic lysosomal degradative pathway, Mel 501 and MST-L cells were treated with A3, A4 or with HRG for 1 h at 37°C in the presence of LysoTracker-Red to identify the lysosomal compartment. In HRG-treated cells, co-incubation with A2 mAb was performed to follow the internalizing ErbB3. Quantitative immunofluorescence analysis clearly demonstrated colocalization of ErbB3-bound A3 and A4 mAbs with the LysoTracker marker in intracellular, perinuclear dots corresponding to lysosomes, while no colocalization was observed with ErbB3 internalized following HRG treatment (**Fig. 6**).

In order to verify whether the ErbB3 targeting to the endocytic degradative pathway induced by A3 and A4 would actually correspond to efficient receptor degradation, we analyzed the kinetics of ErbB3 degradation in MST-L cells treated with HRG or with A2, A3 or A4 mAbs at 37°C for different time points. Western blot analysis using anti-ErbB3 antibodies showed a drastic decrease of the band already after 1 h of treatment with A3 or A4 mAbs, remaining at very low levels at 8 and 24 h of incubation, while upon HRG stimulation, the receptor band was only slightly decreased after 1 and 8 h of stimulation but was completely recovered after 24 h (**Fig. 7**). In contrast, the band

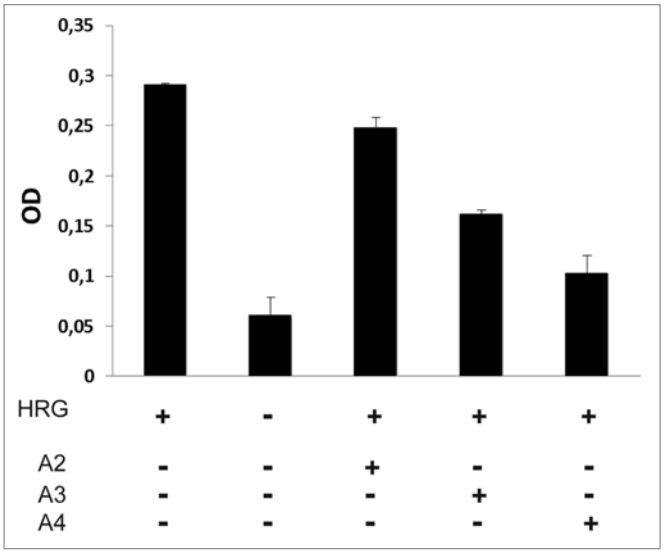

Figure 4. MST-L cells were seeded on 8 μm pored membrane, pretreated with anti-ERBB3 mAbs as above and then stimulated with HRG in the presence of mAbs for 24 h. Quantification of cell migration was performed by densitometric analysis of the filters, after counterstaining with WST-1 and ELISA analysis as reported in materials and methods. Results are reported in the graph as mean optical density (OD) ± SD. HRG stimulates cell migration and A3 and A4 mAbs strongly interfere with the migrating effect, while A2 mAb does not affect it.

corresponding to ErbB3 remained virtually unaltered upon the treatment with A2 mAb (**Fig. 7**).

It has been recently hypothesized that the mechanism underlying receptor downregulation induced by some anti-ErbBs monoclonal antibodies might be consistent also with the inhibition of receptor recycling to the cell surface.[23] To ascertain whether A3 and A4 mAbs might also induce ErbB3 downregulation by interfering with the receptor recycling, MST-L cells were treated at 37°C with A3 or A4 for different time lengths (1, 6 and 24 h). The amount of receptor recycled to the plasma membrane, visualized by re-incubating cells with A3 at 4°C before fixation, was compared with that obtained upon HRG stimulation and quantified by assessing the receptor colocalization with the plasma membrane marker DiI as described above. The results showed that upon incubation for 1 h at 37°C with each antibody or with HRG, no receptor signal on the cell surface was evident (**Fig. 8**), while fluorescent intracellular dots corresponding to the internalized A3-ErbB3 and A4-ErbB3 complexes were clearly visible in permeabilized cells, confirming the antibody-induced receptor internalization (**Fig. 8**, top parts). After incubation for 6 h and 24 h, a progressively increasing receptor signal on the cell surface was evident upon HRG stimulation but not upon A3 and A4 treatment (**Fig. 8**), demonstrating that, differently from the physiological ligand, which is known to induce ErbB3 recycling to the cell surface,[26] the two mAbs are able to downregulate ErbB3 not only by inducing its degradation, but also by blocking its physiological recycling.

Anti-ErbB3 mAbs A2 and A3 recognize two different binding sites. The experiments described above show that there are two distinct categories of ErbB3 receptor binding antibodies; the first is represented by A3 and A4, which are able to block ligand-induced signal transduction, cell proliferation and migration to induce receptor degradation and block its recycling, the second represented by A2, which is unable to inhibit ligand-induced signaling and to induce receptor internalization and degradation. In order to better dissect the molecular basis of their different biological behavior, we decided to map the receptor binding sites recognized by the A2 and A3 antibodies, respectively.

To determine the epitopes, an ELISA-based epitope binding assay was set up.[27] For this purpose, a library of 15mer peptides overlapping by 11 residues and covering the entire ErbB3 extracellular region was built, and individual peptides were plated on a 96-well plate, using as control the entire ErbB3 recombinant extracellular domain, and subjected to an Elisa assay using A2, A3 and A4. The results are shown in **Figure 9**. Quantitative analysis of signal intensity (**Fig. 9A–C**) showed that mAbs A2 and A3 recognize different epitopes contained within peptide CWG PGP GQC LSC RNY the A2 mAb and within peptide QCN GHC FGP NPN QCC the A3 mAb, respectively. No signal was detected for A4, thus suggesting indirectly that this is a complex epitope. Interestingly, the two recognized epitopes are located in two structurally distinct regions of the protein;[28] in fact, while the A2 epitope is located in sub-domain 4, closer to the juxtamembrane region, the A3 epitope is located within domain 2 (**Fig. 9B–D**).

Discussion

Activation of receptor tyrosine kinases (RTKs) has been hypothesized to play a role in the development of melanoma. Among RTKs, several members of the ErbB receptor family are either frequently upregulated (EGFR and ErbB3) or mutated (ErbB4) in melanoma.[29] A further demonstration of the involvement of this receptor family in melanoma cell proliferation derives from the observation that the RTK inhibitor gefitinib partially decreases proliferation of human melanoma cells.[30] Furthermore, in a recent study, Renscke and coworkers have shown by tissue microarray analysis on a large set of tumor samples that ErbB3 expression may serve as a prognostic marker, because its expression correlates with cell proliferation, tumor progression and reduced patient survival.[31] Moreover, the same study has shown that suppression of ErbB3 expression by RNA interference reduces melanoma cell proliferation, migration and invasion in vitro. Hence, we have focused our attention on ErbB3 in a subset of human melanoma cells that co-express ErbB1, ErbB4 and ErbB3 and that are apparently devoid of detectable amounts of ErbB2, and have analyzed in these cells the mechanism of action of a set of anti-human ErbB3 monoclonal antibodies recently generated by our group.[22]

We confirm that melanoma cells are responsive to ligands of the ErbB family, such as heregulin, and show that ligand-dependent activation of AKT signaling, proliferation and migration can be fully abrogated by a subset of our anti ErbB3 monoclonals. Interestingly, not all the anti-ErbB3 antibodies in our collection are able to block heregulin-induced signaling, because one of them, namely, A2, is unable to inhibit ligand-induced

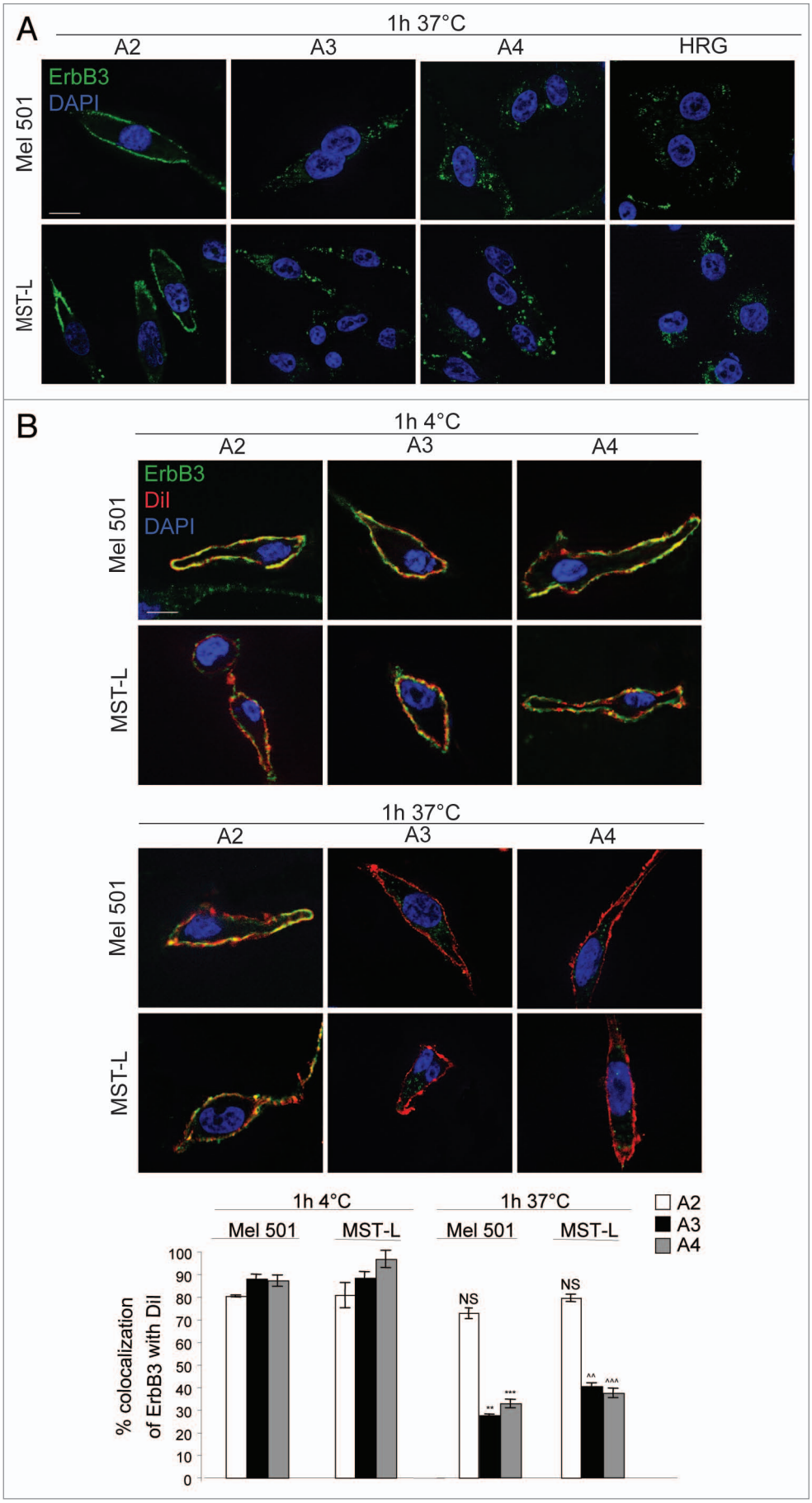

Figure 5. (A) Mel 501 and MST-L cells were treated with A2, A3 or A4 mAbs for 1 h at 37°C before fixation to allow endocytosis of antibody-receptor complexes. Immunofluorescence analysis was performed using a secondary anti-mouse FITC antibody. In both cell lines the signal of A3 or A4 antibodies appears clustered in endocytic dots while the signal of A2 antibody remains uniformly distributed on the plasma membrane. Upon co-incubation of A2 and HRG the signal of A2 mAb appears intracellular as a consequence of the ligand-dependent internalization of ERBB3. Bar = 10 μm. (B) Mel 501 and MST-L cells were incubated with each of the mAbs for 1 h at 4°C, to permit only the binding on the plasma membrane or for 1 h at 37°C, as above, to allow the possible uptake of the receptor-antibody complexes from the cell surface. The plasma membranes were visualized using the Vybrant DiI, as reported in materials and methods. Quantitative immunofluorescence analysis of the percentage of colocalization of the two signals on the plasma membranes was performed by serial optical sectioning and 3D reconstruction as described in materials and methods. Results are expressed as mean values ± SE (standard errors): the percentage of colocalization was calculated analyzing a minimum of 50 cells for each treatment randomly taken from three independent experiments. Student's t-test was performed and significance level has been defined as described in materials and methods. Upon incubation at 4°C, the signal of all three mAbs colocalizes with DiI at the plasma membrane. Upon incubation at 37°C, the colocalization of A3 and A4 with DiI is strongly reduced; the slight decrease of colocalization of A2 mAb with the plasma membrane marker at 37°C compared with the binding at 4°C is not significant. **$p < 0.001$ vs. the corresponding cells treated with A3 at 4°C, ***$p < 0.0001$ the corresponding cells treated with A4 at 4°C, ^^$p < 0.0001$ vs. the corresponding cells treated with A3 at 4°C, ^^^$p < 0.0001$ vs. the corresponding cells treated with A4 at 4°C. Bar = 10 μm.

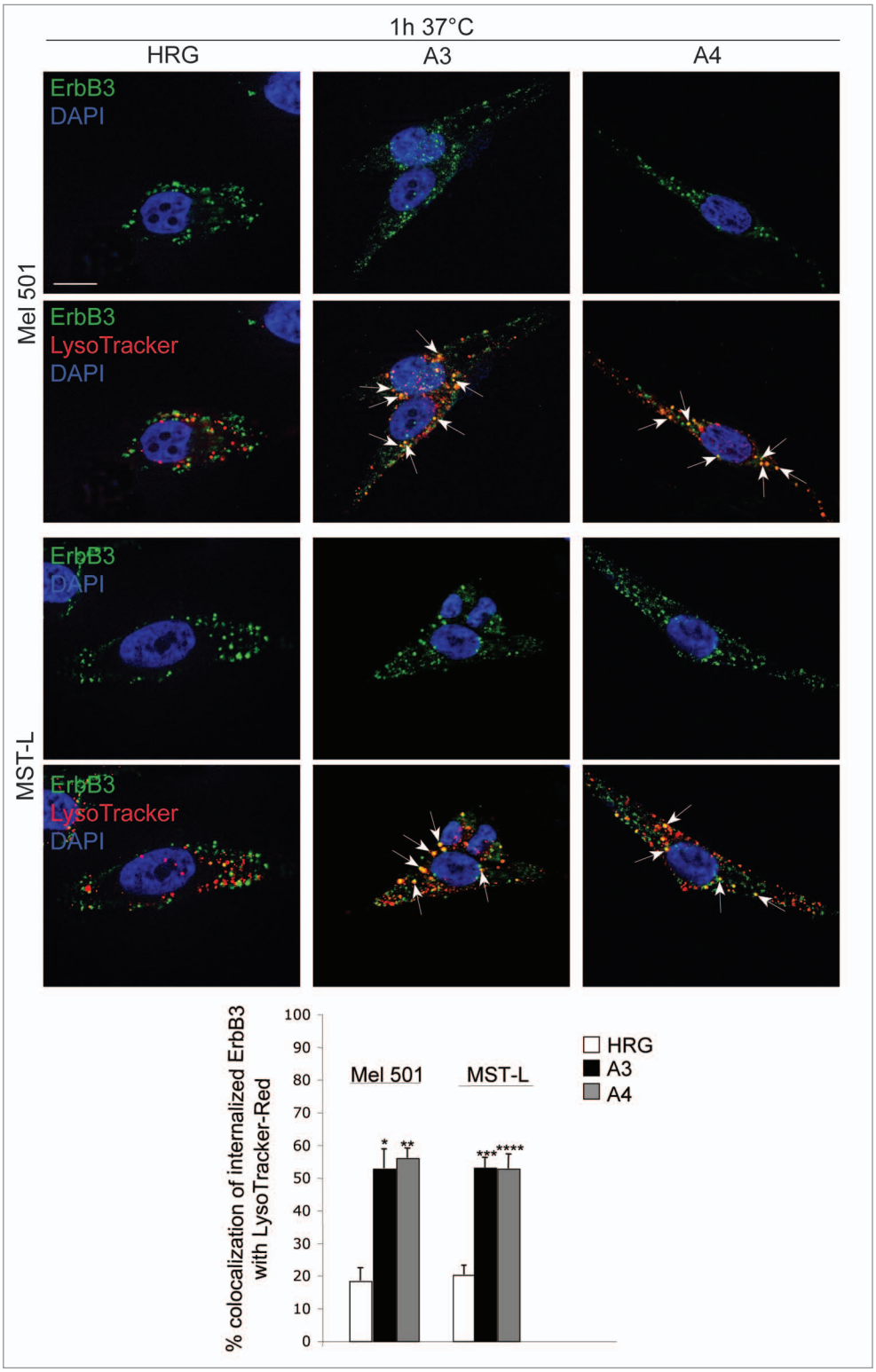

Figure 6. Mel 501 and MST-L cells were treated with A3, A4 or co-incubated with HRG and A2 mAb for 1 h at 37°C in the presence of LysoTracker-Red as reported in materials and methods. Quantitative immunoflorescence analysis of the percentage of colocalization of signals and 3D reconstruction was performed as described in materials and methods. Results are expressed as mean values ± SE (standard errors): the percentage of colocalization was calculated analyzing a minimum of 50 cells for each treatment randomly taken from three independent experiments. Student's t-test was performed and significance level has been defined as described in materials and methods. A colocalization of ERBB3-bound A3 and A4 mAbs with the LysoTracker marker in intracellular, perinuclear dots corresponding to lysosomes is evident; no colocalization is observed between the lysosomal marker and ERBB3 internalized following HRG treatment. *$p < 0.0001$ vs. the corresponding HRG-treated cells, **$p < 0.0001$ vs. the corresponding HRG-treated cells, ***$p < 0.0001$ vs. the corresponding HRG-treated cells, ****$p < 0.0001$ vs. the corresponding HRG-treated cells. Bar = 10 μm.

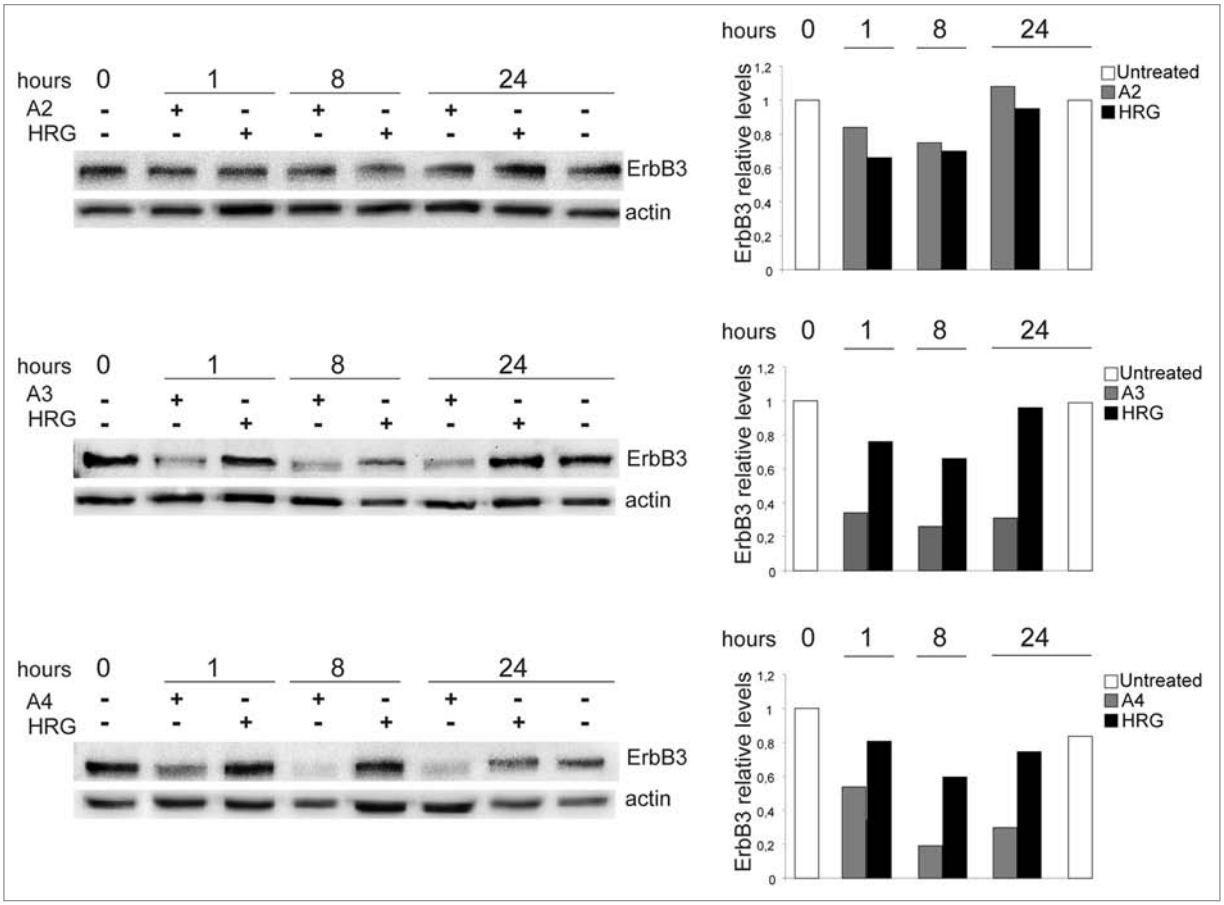

Figure 7. Western blot analysis using anti-ERBB3 polyclonal antibodies in MST-L cells treated with HRG or with A2, A3 or A4 mAbs at 37°C for the different time points (1, 8 and 24 h). A drastic decrease of the band corresponding to ERRB3 is evident after 1 h of treatment with A3 or A4 mAbs and persists at 8 and 24 h of incubation while upon HRG stimulation the receptor band is slightly decreased after 1 and 8 h of stimulation, and is recovered after 24 h. The band corresponding to ERBB3 remains virtually unaltered upon the treatment with A2 mAb. The equal loading was assessed with anti-actin or antibody and densitometric analysis was performed as described in materials and methods.

ErbB3 signaling in spite of a similar receptor binding affinity as compared with the "active" A3 and A4 monoclonals. This different behavior prompted us to gain further insights into the mechanism of action of active vs. non-active anti-ErbB3 antibodies.

Our main observation was that activity correlated with receptor downregulation. When compared with heregulin, the ability of A3, and in particular A4, to downregulate ErbB3 receptor levels was dramatic; while heregulin was capable to slightly decrease receptor levels up to residual 60% after 8 h of treatment, mAbs A3 and A4 led to residual 21 or 17% receptor levels in the same conditions. In contrast, the inactive A2 monoclonal was unable to reduce receptor levels. When we analyzed the reasons of this discrepancy with a series of localization studies, we observed that all three antibodies bound ErbB3 receptor on the plasma membrane with the same efficiency in conditions of blockade of receptor internalization, but only the two active antibodies triggered receptor internalization at 37° C. Interestingly, while A2 was unable to induce receptor internalization by itself, it did not block receptor internalization induced by heregulin, in line with its inability to compete with the ligand for binding to the receptor.

Another set of interesting observations comes from the comparison of receptor re-localization induced by heregulin or by the two active antibodies. Upon cell exposure to heregulin, ErbB3 was clearly associated with intracellular vesicles that did not stain positive for the lysosomal dye lysotracker. This is in line with previous observations showing that ErbB3 is inefficiently sorted to the degradation pathway.[32] This was confirmed by our finding that sometime after exposure to the ligand, ErbB3 is recycled to the membrane surface. In contrast, antibodies A3 and A4 induced sorting of ErbB3 into lysosomes and blocked receptor recycling back to the cell surface. Previous studies conducted with EGFR/ErbB3 receptor chimeras led us to postulate that, unlike ErbB1, which is naturally destined to lysosomal degradation through a ligand-induced mechanism that involves recruitment of c-Cbl and receptor poly-ubiquitination, the C-terminal tail of ErbB3 is unable to recruit c-Cbl and shunts ErbB3 to the recycling pathway.[32] This conclusion is, at least in part, contradicted by our evidence that it is possible to induce ErbB3 receptor polyubiquitination and lysosomal degradation, but this depends upon the type of receptor binders. In fact we show that ErbB3 can undergo alternative fates upon internalization: (1) recycling

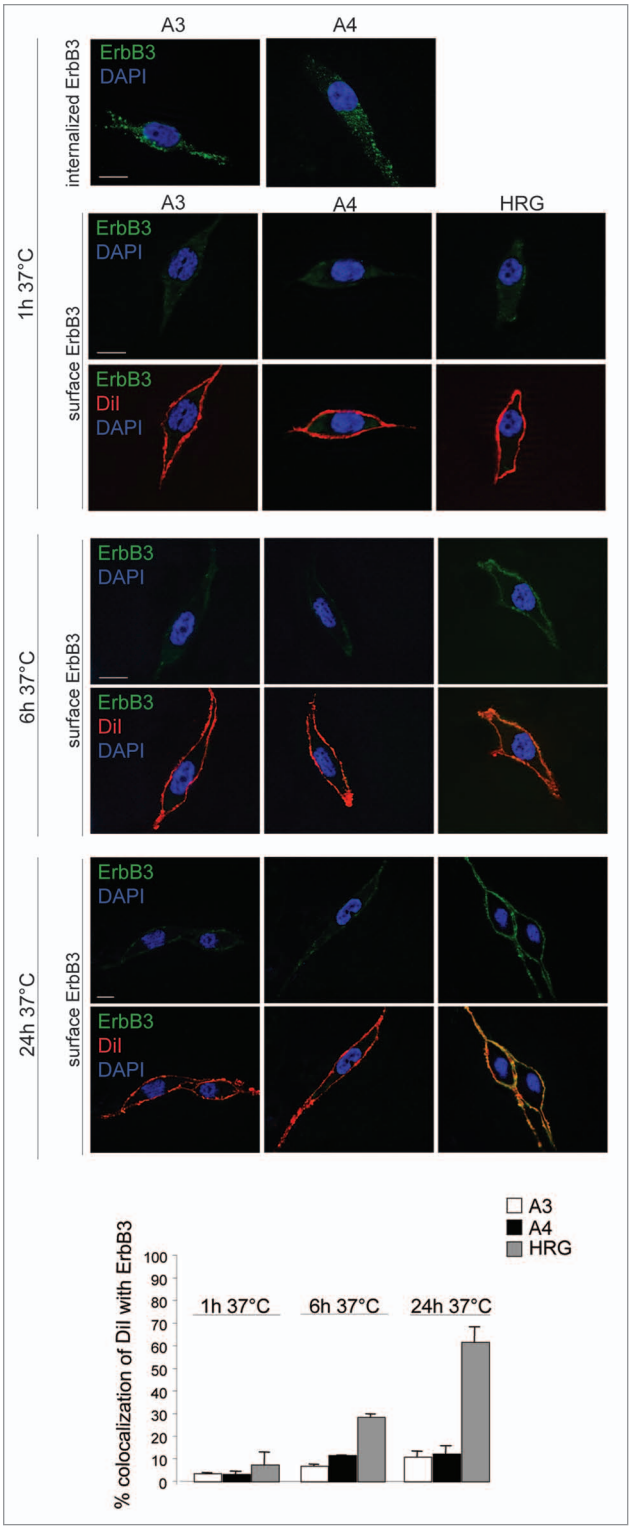

Figure 8. MST-L cells treated at 37°C with A3 or A4 or HRG for different times (1, 6 and 24 h) were fixed and permeabilized (top part) or alternatively re-incubated with A3 and DiI for 1 h at 4°C before fixation. Quantitative immunofluorescence analysis of the percentage of colocalization of plasma membrane signals and 3D reconstruction was performed as described in materials and methods. Results are expressed as mean values ± SE (standard errors): the percentage of colocalization was calculated analyzing a minimum of 50 cells for each treatment randomly taken from three independent experiments. Upon incubation for 1 h at 37°C with mAbs or with HRG, no receptor signal on the cell surface is detectable while intracellular dots corresponding to the internalized A3-ERBB3 and A4-ERBB3 complexes are visible in permeabilized cells (top part). Upon 6 h and 24 h of incubation, a progressively increasing receptor signal on the cell surface was evident upon HRG stimulation; no plasma membrane receptor signal is evident upon 6 h and 24 h of A3 and A4 treatment. Bars: 10 μm.

to the cell membrane when the receptor is engaged by its natural ligands such as heregulin; (2) lysosomal sorting and consequent degradation when engaged by non-natural ligands such as the A3 and A4 monoclonal antibodies described here. The E3 ubiquitin ligase Nrdp1 has been postulated to contribute to the maintenance of steady state ErbB3 levels by mediating its ligand-independent degradation.[33] In follow-up studies, it will be important, therefore, to determine if the monoclonal antibody-induced massive degradation of ErbB3 also involves the activity of the Nrdp1 E3 ligase, or if is due to de novo recruitment of other players such as c-Cbl.

Antibodies directed against the extracellular region of ErbB receptors have played important roles in our understanding of their mechanism of activation. Crystal structures of the Fab fragments from several different inhibitory antibodies in complex with the extracellular region of EGFR, ErbB2 and, very recently, ErbB3 have been determined and highlight different modes of binding and mechanisms of receptor inhibition.[21,34] In this context, the epitope mapping studies performed with two of our monoclonals allow us to establish an indirect comparison with the binding mode of clinically relevant monoclonals against other receptors of the same family and to make the following considerations. A2 binds to an epitope located within domain 4, which makes its mode of binding similar to that of the anti-ErbB2 mAb trastuzumab (Herceptin™).[28] Trastuzumab activity is largely restricted to breast cancers with the highest level of HER2 overexpression, while in cancers that have low or moderate HER2 expression, this monoclonal antibody is not active.[35] Indeed trastuzumab does not impair signaling and growth in vitro of cells expressing moderate amounts of HER2-ErbB3 heterodimers (our data unpublished). Likewise, A2 is inactive both in HER2-ErbB3 cells (our data unpublished) and in EGFR-ErbB3-expressing cells such as MST-L and Mel 501 studied in the present work. Differently from A2, the anti-ErbB3 monoclonal A3 binds to an epitope located within domain 2, which makes its mode of binding similar to that of the anti-HER2 mAb pertuzumab.[36] Like pertuzumab, A3 shares the property of being a potent inhibitor of signal transduction and an inducer of receptor downregulation, also in conditions of low to moderate receptor expression. Based on this similarity, we expect A3 to be active in a variety of cancers, where ErbB3 is involved as a key node in PI3K/Akt signal transduction through the upregulation of heterodimers with EGFR, ErbB2, ErbB4 or other non-ErbB family RTKs. This finding is of particular relevance in light of the growing interest in the ErbB3/PI3K node as a potential target for anticancer therapy, which has led to the development of several anti-human ErbB3 monoclonal antibodies.[19-21] Interestingly, if we compare the mode of biding of our anti-ErbB3 antibody

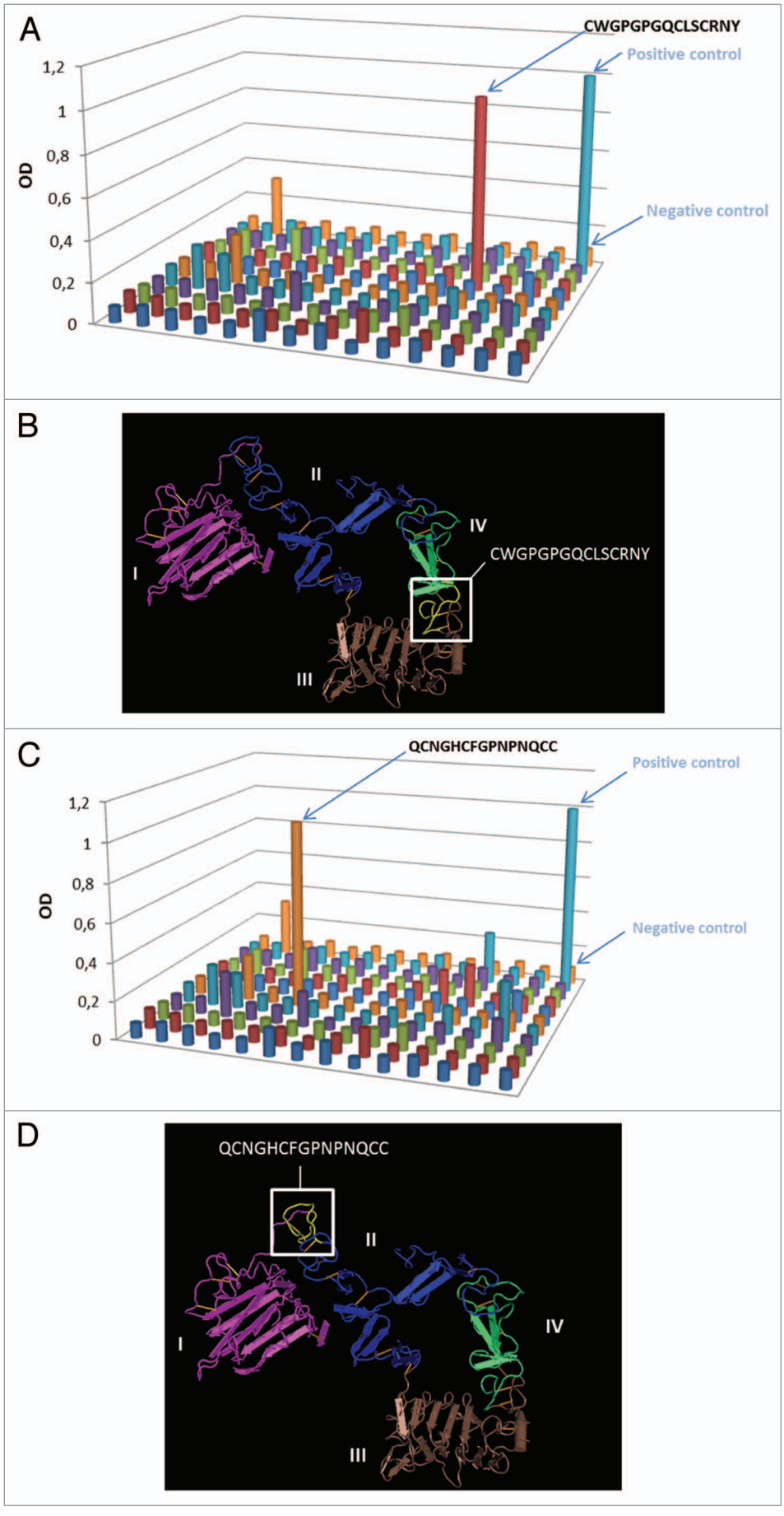

Figure 9. Epitope mapping of A2 and A3 antibodies. 96 well plates were coated with 15mer peptides overlapping by 11 residues and covering the extracellular region (ECD) of human ErbB3. Antibodies were incubated for 2 h at 1 μg/ml and an anti-mouse IgG HRP-conjugated antibody was used for detection. (A) A2 epitope mapping. Optical density at 450 nm for each single well is reported. Peptide 128, corresponding to CWG PGP GQC LSC RNY (aa 509–523 of human ErbB3 protein, accession: P21860) showed a strong signal, comparable to that of recombinant human ErbB3 protein (positive control). No significant signal was observed neither with a CEA unrelated peptide (negative control) nor with other human ErbB3 peptides. (B) Diagram of the crystal structure of the extracellular region of human ErbB3 generated by Cn3D software (NCBI). Domain I is shown in purple, domain II in Blue, domain III in brown and domain IV in green. The epitope is indicated in yellow and maps within domain IV. (C) A3 epitope mapping shows a specific signal for peptide 54 (QCN GHC FGP NPN QCC, aa 213–228). Optical density at 405 nm is indicated. (D) Position of the epitope within ErbB3 crystal structure. $_{213}$QCN GHC FGP NPN QCC$_{228}$ maps within the finger-like projection of domain II, mediating the inter-receptor dimeration.

A3 with that of MM-121[19] or of the dual anti-EGFR/anti-ErbB3 antibody MEHD7945A,[21] A3 differs significantly from the other two because of its unique pertuzumab-like mode of binding.

It has been shown that combining pairs of anti-HER2 mAbs recognizing distinct epitopes exerts a superior antitumor effect compared to the use of individual antibodies.[25] Since the effect is seen both in vivo and in vitro, it is likely that this is not due to the involvement of immunological mechanisms but to a more powerful down-regulation of the signaling survival pathways of HER2-expressing cancer cells exerted by the antibodies combinations. Interestingly, the synergistic effect has been best observed when an anti-HER2 monoclonal recognizing the dimerization site of HER2 is combined with a "non-active" antibody that recognizes a different domain of the HER2 extracellular region and has been attributed to the formation

of lattices that more potently affect receptor internalization and routing to degradation. In the light of our epitope binding studies, it will be of relevance to assess the combinatorial effects of our anti-ErbB3 monoclonals among themselves or also in association with anti-EGFR or anti-HER2 antibodies in different experimental settings in order to identify more powerful anticancer treatments.

Materials and Methods

Cells and treatments. Human melanoma cell lines Mel 501[37] and MST-L (kindly provided by Dr. Armando Bartolazzi, Ospedale Sant'Andrea) were cultured in RPMI supplemented with 10% FBS. To induce ErbB3 signaling, cells were stimulated with 30 ng/ml HRG-β1 (HRG) for 10 min at 37°C. To interfere with ErbB3 signaling, cells were pre-incubated with 10 μg/ml of A2, A3 or A4 monoclonal antibodies for 1 h at 37°C before HRG stimulation. To induce the binding of anti-hErbB3 monoclonals exclusively to the plasma membrane, cells were incubated with the antibodies for 1 h at 4°C. To analyze the possible internalization, degradation and recycling of ErbB3, cells were stimulated with HRG 100 ng/ml or incubated with A2, A3 and A4 at 37°C for different times as indicated. For the visualization of the cell surface, plasma membranes were labeled with Vybrant DiL cell labeling solution for 1 h at 4°C before fixation. To induce LysoTracker internalization, cells were incubated with LysoTracker-Red for 1 h at 37°C and then fixed. For proliferation assays, cells were incubated with the anti-hErbB3 monoclonals for 1 h at 37°C and then stimulated with 30 ng/ml HRG for 48 or 72 h. For migration assays, cells were seeded in 6-well plates and they were serum starved in medium containing 0.1% FCS for 24 h. Cells were incubated with 100 μg/mL of antibodies for 1 h, and 8,000 cells were then seeded either onto a membrane with 8 μm pores of a Boyden chamber (BD Biosciences) containing 50 μL serum-free medium. RPMI medium with 100 ng/ml of HRG was used as a chemoattractant in the lower chamber. The cells were allowed to migrate for 24 h. Migrated cells were stained with WST-1 (ROCHE) and analyzed using an ELISA plate reader. The data are shown as mean ± SD. Western blots were performed as described in reference 22.

Antibodies and reagents. Antibodies against ErbB2, ErbB4, phospho-ErbB3 and phospho-Akt were purchased from Cell Signaling Technology. Anti-EGFR, -ErbB3, -Akt antibodies were obtained from Santa Cruz Biotechnology. Anti-rabbit were purchased from AbCam. The rabbit anti-Ki67 polyclonal antibodies were from Zymed Laboratories. FITC-conjugated goat anti-mouse IgG was obtained from Cappel Research Products. Texas Red-conjugated goat anti-rabbit IgG were from Jackson Immunoresearch Laboratories. DAPI was purchased from Sigma. Human HRG-1-β1 (HRG) was purchased from R&D Systems. Vybrant DiI cell labeling solution was from Invitrogen. LysoTracker-Red was obtained from Molecular Probes.

Immunofluorescence analysis. Melanoma cells, grown on coverslips and incubated with the monoclonals A2, A3 and A4 and/or stimulated with HRG as described above were fixed with 4% paraformaldehyde in PBS for 30 min at 25°C followed by treatment with 0.1 M glycine for 20 min at 25°C and with 0.1% Triton X-100 for an additional 5 min at 25°C to allow permeabilization. To evaluate cell proliferation, cells were then incubated for 1 h at 25°C with the rabbit anti-Ki67 polyclonal antibodies. The primary antibodies were visualized using goat anti-mouse IgG-FITC and goat anti-rabbit IgG-Texas Red for 30 min at 25°C. Nuclei were stained with DAPI. Coverslips were finally mounted with mowiol for observation. To assess the extent of colocalization of fluorescence signals, cells were scanned in a series of 0.5 μm sequential sections with an ApoTome System (Zeiss) connected with an Axiovert 200 inverted microscope (Zeiss); image analysis was then performed by the Axiovision software and 3D reconstruction of a selection of three central out of the total number of the serial optical sections was shown in each figure. Quantitative analysis of the extent of colocalization was performed using Zeiss KS300 3.0 Image Processing system. The mean ± standard error (SE) percent of colocalization was calculated, analyzing a minimum of 50 cells for each treatment randomly taken from three independent experiments. Percentage of Ki67-positive cells was obtained counting for each treatment a total of 500 cells, randomly observed in 10 microscopic fields from three different experiments. Results have been expressed as mean values ± standard errors (SE). p-values were calculated using Student t-test, and significance level has been defined as $p < 0.05$.

Epitope mapping. A human ErbB3 PepTrack Peptide Library was purchased from JPT (Jerini Peptide Technologies GmbH). Peptides were 15-mers overlapping by 11 residues and covered the entire extracellular region of the mature human ERBB3 receptor. 96-well plates were coated with the peptides and antibody binding was assessed as described in reference 27.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental material can be found at:
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