3. RESULTS

3.1. Consistent expression of DR5 but selective expression of DR4 in melanoma cells lines

For obtaining an overview about the expression level of TRAIL death receptors, FACS analyses for DR4 and DR5 were performed on a panel of 7 human melanoma cell lines and 3 cultures of normal human melanocytes (NHM). Whereas surface expression of DR5 was consistently found in all melanoma cell lines analyzed, significant DR4 expression was seen in only two of them (A-375 and SK-Mel-13). Normal melanocytes showed only weak expression of DR5 and were negative for DR4 (Fig. 3.1.).

The FACS data were confirmed by Western blot analyses of the 7 melanoma cell lines and of 5 NHM. In all investigated cell lines, the DR5 antibody identified two protein bands corresponding to 40 kDa and 47 kDa molecular weight with a tendency to upregulation in melanoma cells. The DR4 antibody revealed several weak protein bands between 50 and 60 kDa, which were ubiquitously expressed and regarded as unspecific. Only a 44 kDa protein obtained in A-375 as well as in SK-Mel-13 corresponded well with the positive FACS data (Fig. 3.2.a).

To demonstrate specificity of the identified protein bands, SK-Mel-13 was transiently transfected with plasmids containing full length cDNA of DR4 or DR5. Strong increase in the 44 kDa protein after transfection with DR4 as well as of the 47 kDa protein after transfection with DR5 proved the identity of these protein bands for the respective death receptor in melanoma cells (Fig. 3.2.b).

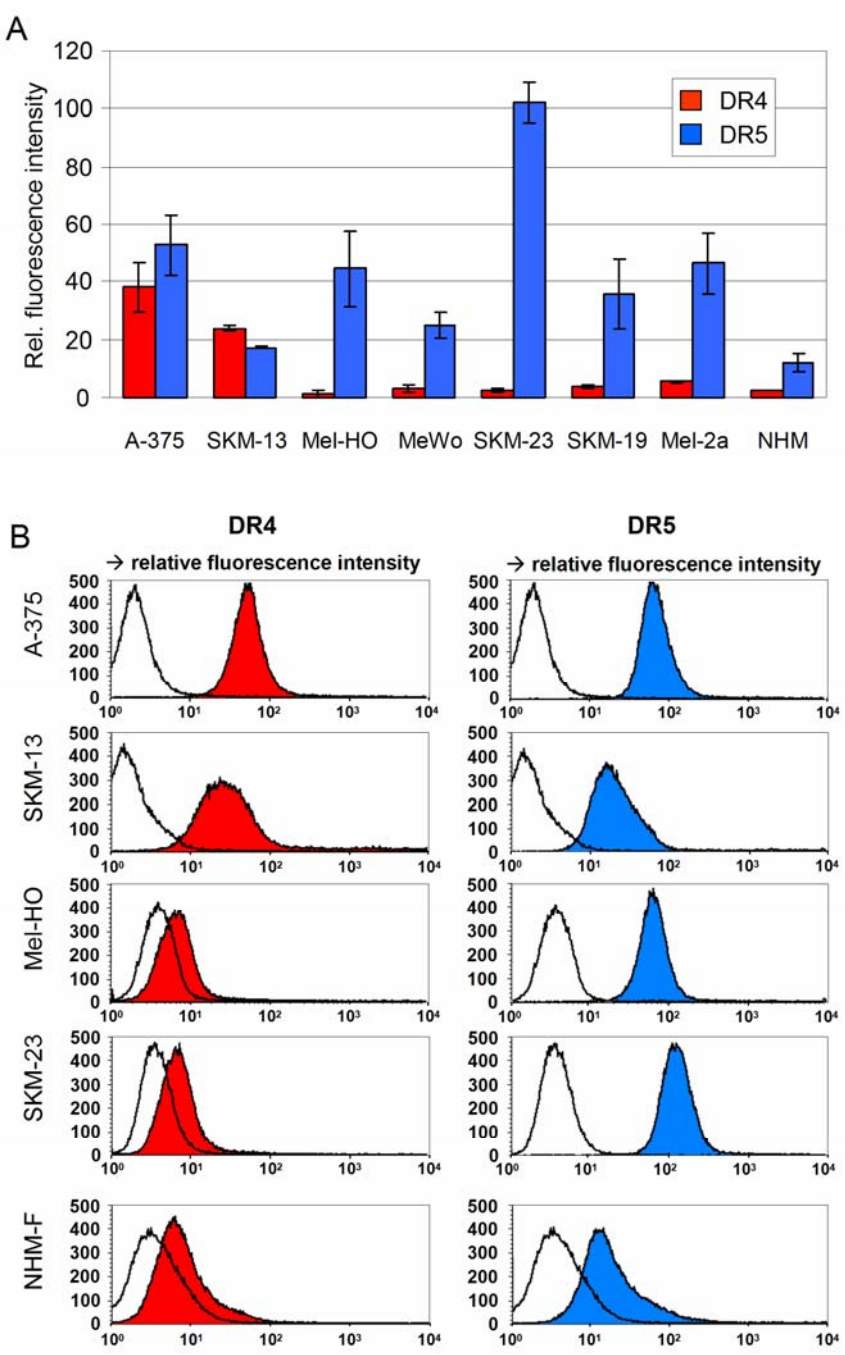

Figure 3.1. Consistent expression of DR5 but not DR4 in melanoma cell lines by FACS

(A) Surface expression of DR4 and DR5 was analysed by FACS analysis in 7 human melanoma cell lines and in cultures of normal human melanocytes (NHM). Median values obtained for melanoma cell lines (two independent experiments) and for NHM (three independent cultures) were normalized with respect to the negative control (primary antibody omitted). Red bars are representative of DR4 and blue bars of DR5.
(B) Examples of DR4-positive cell lines (A-375, SK-Mel-13) and of DR4-negative cells (Mel-HO, SK-Mel-23) as well as of NHM are given. Cells were labelled with specific monoclonal TRAIL-R1/DR4 and TRAIL-R2/DR5 antibodies (red and blue graphs, respectively) as well as with an isotypic control IgG_1 mouse monoclonal antibody (open graphs). A shift to the right side indicates death receptor surface expression.

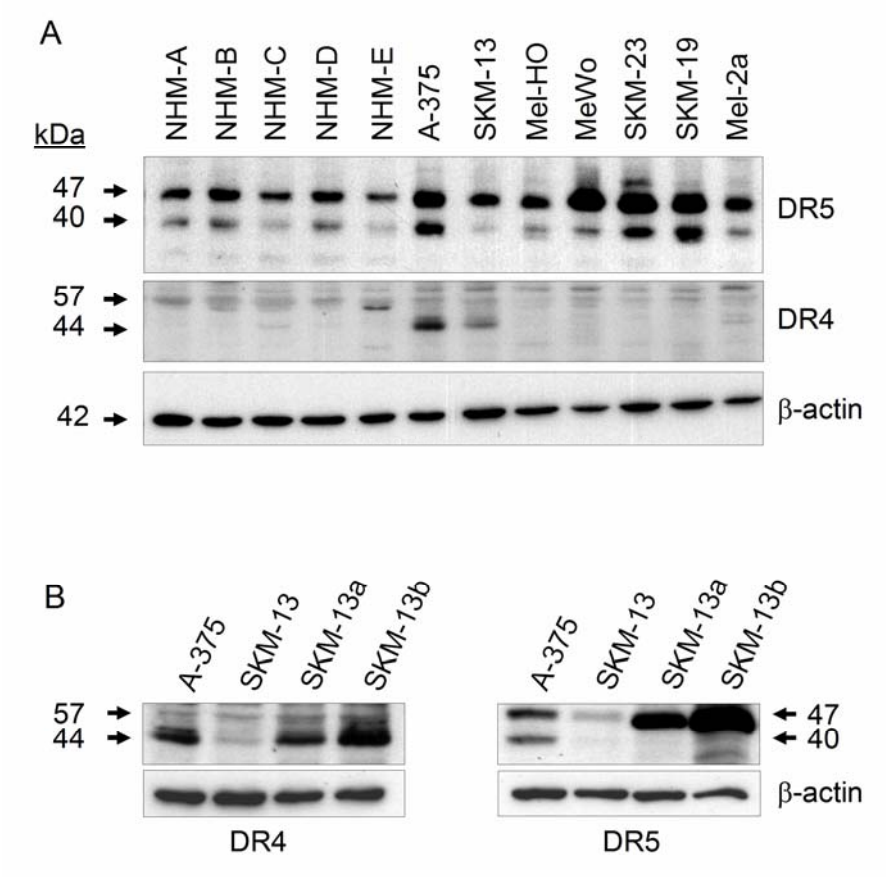

Figure 3.2. Strong evidence of identified protein bands for DR4 and DR5 by transient transfection

A) Preparations of total protein of 7 melanoma cell lines and of 5 cultures of normal human melanocytes (NHM) were analysed by Western blotting for DR4 and DR5 expression. Equal protein amounts (25 mg per lane) were separated by SDS-PAGE. Consistent blotting was proven by ponceau staining and by evaluation of β-actin expression. Arrows indicate molecular weights of identified protein bands. A highly comparable pattern of protein bands was obtained in a second experiment started from independently grown cell cultures.
B) SK-Mel-13 cells transiently transfected with two different concentrations of DR4 and DR5 expression plasmids (SKM-13a: 0.1µg/ml; SKM13b: 0.2µg/ml) were investigated 48 h after transfection by Western blot analysis for DR4 and DR5 (SDS-PAGE, 40 mg total protein per lane). Expression was compared to basic expression in untransfected SK-Mel-13 and A-375 cells. Two independent series of transfection experiments have been performed which revealed the same result.

3.2. High sensitivity to TRAIL-mediated apoptosis in DR4-positive melanoma cell lines

For investigating possible correlations between receptor expression and sensitivity to TRAIL-induced apoptosis, relative apoptosis values were determined by measuring DNA fragmentation. DNA fragmentation was measured photometrically using an enzyme-linked immunoassay for determination of cytoplasmic histone-associated DNA fragments. Cells were analyzed at three different times (6 h, 24 h and 48 h) after addition of TRAIL (20 ng/ml and 100 ng/ml). Both DR4-positive melanoma cell lines (A-375 and SK-Mel-13) showed highest sensitivity already after 6 h, whereas of the 5 melanoma cell lines lacking DR4, only two (Mel-HO and SK-Mel-19) showed a (weaker) apoptotic response at this early time. Apoptosis rates increased after 24 h in Mel-HO and (less) in SK-Mel-19, in clear contrast to A-375 and SK-Mel-13, for which the rate of DNA fragmentation already started to decline after 6 h. The cell lines SK-Mel-23 and Mel-2a as well as normal melanocytes showed a rather limited response detectable only after 48 h, whereas MeWo was completely resistant over the period of observation (Fig. 3.3.). Thus, melanoma cell lines expressing DR4 in addition to DR5 were highly responsive to TRAIL, whereas DR5 alone mediated a weaker and delayed apoptotic response.

3.3. Cytotoxicity follows apoptotic cell death after TRAIL treatment

To determine whether high apoptosis induced by TRAIL in DR4-positive melanoma cell lines was accompanied by nonapoptotic cell death as cytotoxicity, we have measured TRAIL-induced cytotoxicity in parallel with apoptosis. Cells were analyzed at three different times (6 h, 24 h and 48 h) after addition of TRAIL (20 ng/ml and 100 ng/ml). The investigated melanoma cell lines did not show any signs of cytotoxicity at an early time after TRAIL treatment (6 h), indicating that the primary effect of TRAIL was apoptosis in DR4-positive melanoma cell lines. TRAIL-induced cytotoxicity was seen only 24 h after treatment in sensitive melanoma cells. Cytotoxicity was not measured in TRAIL-resistant cells (Fig. 3.4.), clearly demonstrating that the primary effect of TRAIL is apoptosis induction, whereas cytotoxicity is a secondary effect.

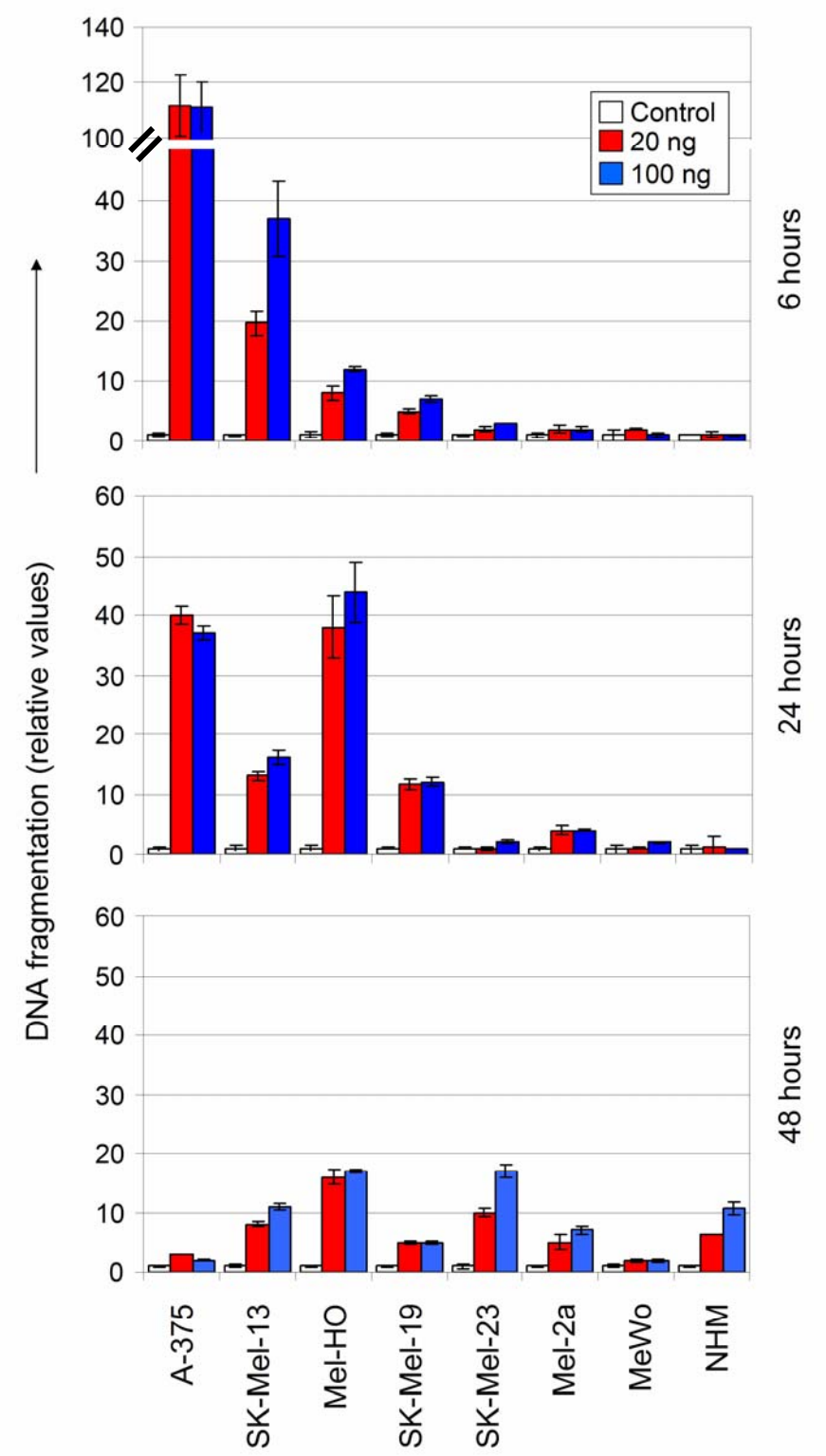

Figure 3.3. High sensitivity of DR4-positive melanoma cells for TRAIL-induced apoptosis

Seven melanoma cell lines as well as normal human melanocytes (NHM) were treated with 20 ng/ml TRAIL (red bars) or with 100 ng/ml (blue bars). After incubation times of 6 h, 24 h and 48 h, relative DNA fragmentation rates indicative of apoptosis was determined. Untreated controls (white) were individually for each cell line set to 1. Mean values and standard deviations of three independent experiments each performed with double values are shown.

3.4. Strong activation of apoptosis cascades in DR4-positive melanoma cells

For obtaining insight into the pathways of TRAIL-mediated apoptosis in melanoma cells with regard to their expression of death receptors, cleavage of caspase-8, -10, -9, -3 and -7, BID, XIAP and DFF45 was monitored in the panel of 7 melanoma cell lines after treatment with 20 ng/ml TRAIL for 4 h. The sensitive melanoma cell lines (A-375, SK-Mel-13, Mel-HO and SK-Mel-19) showed caspase-8 cleavage into its intermediate and final products (43/41 kDa, 18 kDa) (Fig. 3.5.). Expression of caspase-10 was identified in all investigated melanoma cell lines except of MeWo at molecular weights of 59, 55 and 30 kDa likely corresponding to isoforms described previously in lymphoma cells (Sprick *et al*, 2002). Degradation of caspase-10 after TRAIL treatment was significant in A-375, with a similar tendency in SK-Mel-13. In contrast, there was no evidence for caspase-10 cleavage after 4 h in the melanoma cell lines lacking DR4.

The p20 intermediate cleavage product of the main effector caspase-3 was found in all melanoma cell lines after TRAIL treatment. Interestingly, the 17 kDa mature product (Han *et al*, 1997) selectively appeared only in the sensitive cell lines A-375, SK-Mel-13, Mel-HO and SK-Mel-19, which closely correlats with the apoptotic response of the cells 6 h after TRAIL treatment (Fig. 3.5.)., cleavage of caspase-7 and of the death substrate DFF45 occurred largely in parallel with caspase-3 activation. Also, the 29 kDa cleavage product of XIAP was seen in sensitive melanoma cells, most prominently in A-375. However, cleavage of XIAP did not result in a significant reduction of its basic expression after 4 h treatment (Fig. 3.5.).

The basic expression level of initiator caspases seemed to be critical for efficient DISC formation in DR4-negative melanoma cells. Caspase-8 and -10 were expressed in melanoma cell lines to varying degrees, however, sensitive Mel-HO cells lacking DR4 expression were characterized by highest expression levels of both initiator caspases, whereas expression of initiator caspases was rather weak in SK-Mel-13. On the other hand, high caspase-8 or caspase-10 levels were not sufficient for supporting TRAIL sensitivity in SK-Mel-23 and Mel-2a (Fig. 3.5.).

Clear signs for activation of the mitochondrial pathway were seen in A-375 as degradation of the proform of BID and as occurrence of the 37 kDa cleavage product of caspase-9 (Fig. 3.5.). On the other hand, SK-Mel-13 and DR4-negative melanoma cell lines did not reveal any degradation of BID and showed weaker activation of caspases 8 and 9, which may be considered an indication for additional alternative pathways also in TRAIL-induced apoptosis.

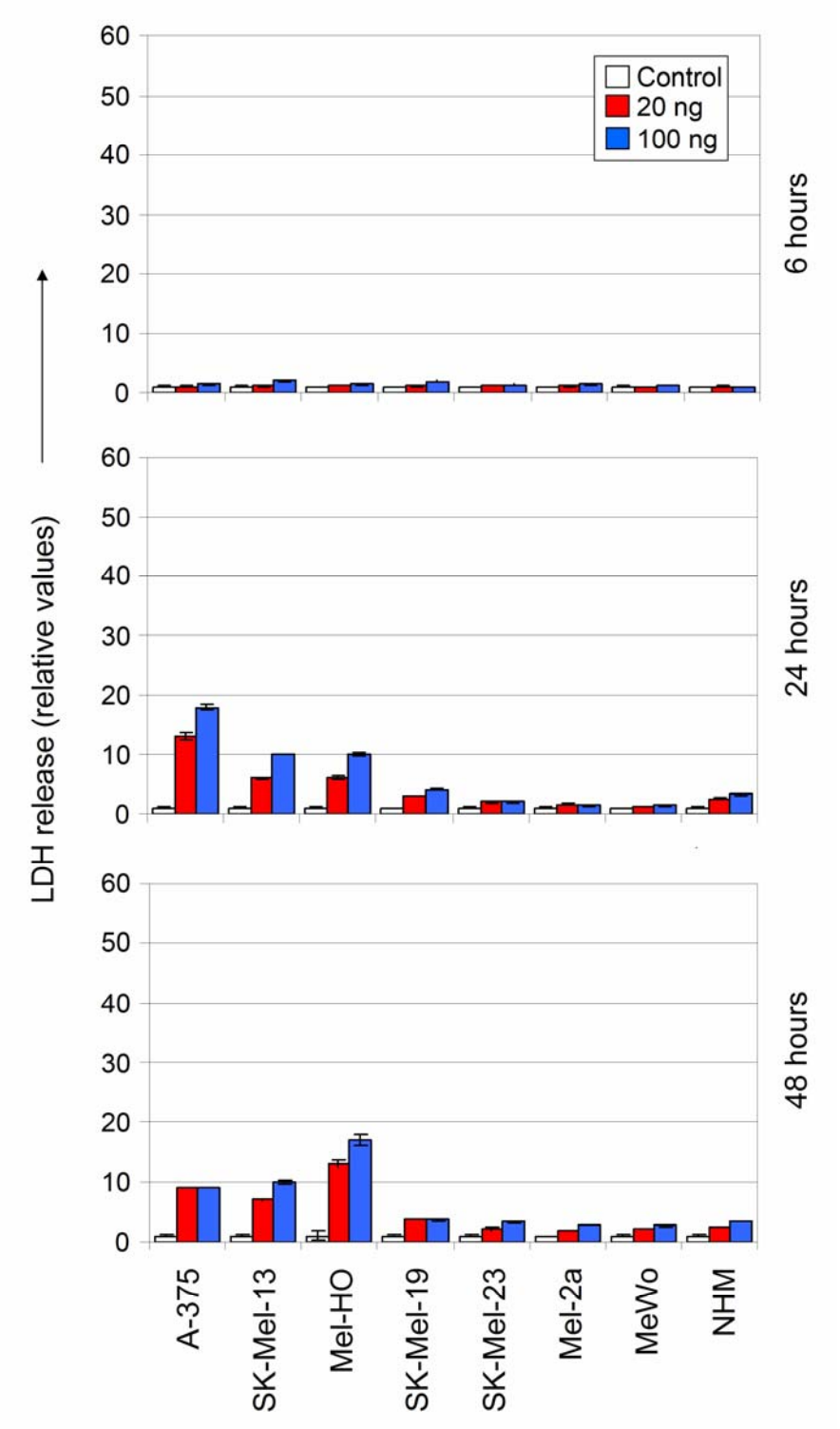

Figure 3.4. Cytotoxic effect of TRAIL in melanoma cells

Seven melanoma cell lines as well as normal human melanocytes (NHM) were treated with 20 ng/ml TRAIL (red bars) or with 100 ng/ml (blue bars). After incubation time (6 h, 24 h and 48 h), relative lactate dehydrogenase activities indicative of cytotoxicity was determined in cell supernatants. Untreated controls (white) were individually for each cell line, which were set to 1. Mean values and standard deviations of three independent experiments each performed with double values are shown.

3.5. Significance of the mitochondrial pathway for TRAIL-induced apoptosis in melanoma cells

For further assessing the implications of the mitochondrial pathway for TRAIL-induced apoptosis in melanoma, stably transfected cell clones of A-375 and Mel-HO highly overexpressing Bcl-2 were evaluated with respect to TRAIL sensitivity. TRAIL-induced apoptosis was completely suppressed by overexpression of Bcl-2 in both cell clones, as compared to mock-transfected cells (Fig. 3.6.a). In SK-Mel-13 cell clones stably transfected with Bcl-x_L functionally related to Bcl-2 (SKM-13-Bcl-x_{L11}, SKM-13-Bcl-x_{L15}), overexpression of Bcl-x_L resulted in a 2.6 $\pm$ 0.3-fold and 2.1 $\pm$ 0.4-fold reduction, respectively, of TRAIL-induced apoptosis as compared to mock-transfected cells (Fig 3.6.c). In parallel with these data, transient transfection of SK-Mel-13 with a Bcl-2 expression plasmid resulted in 1.6 $\pm$ 0.3-fold reduction of TRAIL-induced apoptosis (data not shown). Overexpression of Bcl-2 and Bcl-x_L was determined by Western blot analysis (Fig. 3.6.b,d) Thus, the mitochondrial pathway seemed to exert at least contributory functions for TRAIL-induced apoptosis in SK-Mel-13, whereas it was essential in A-375 and in Mel-HO.

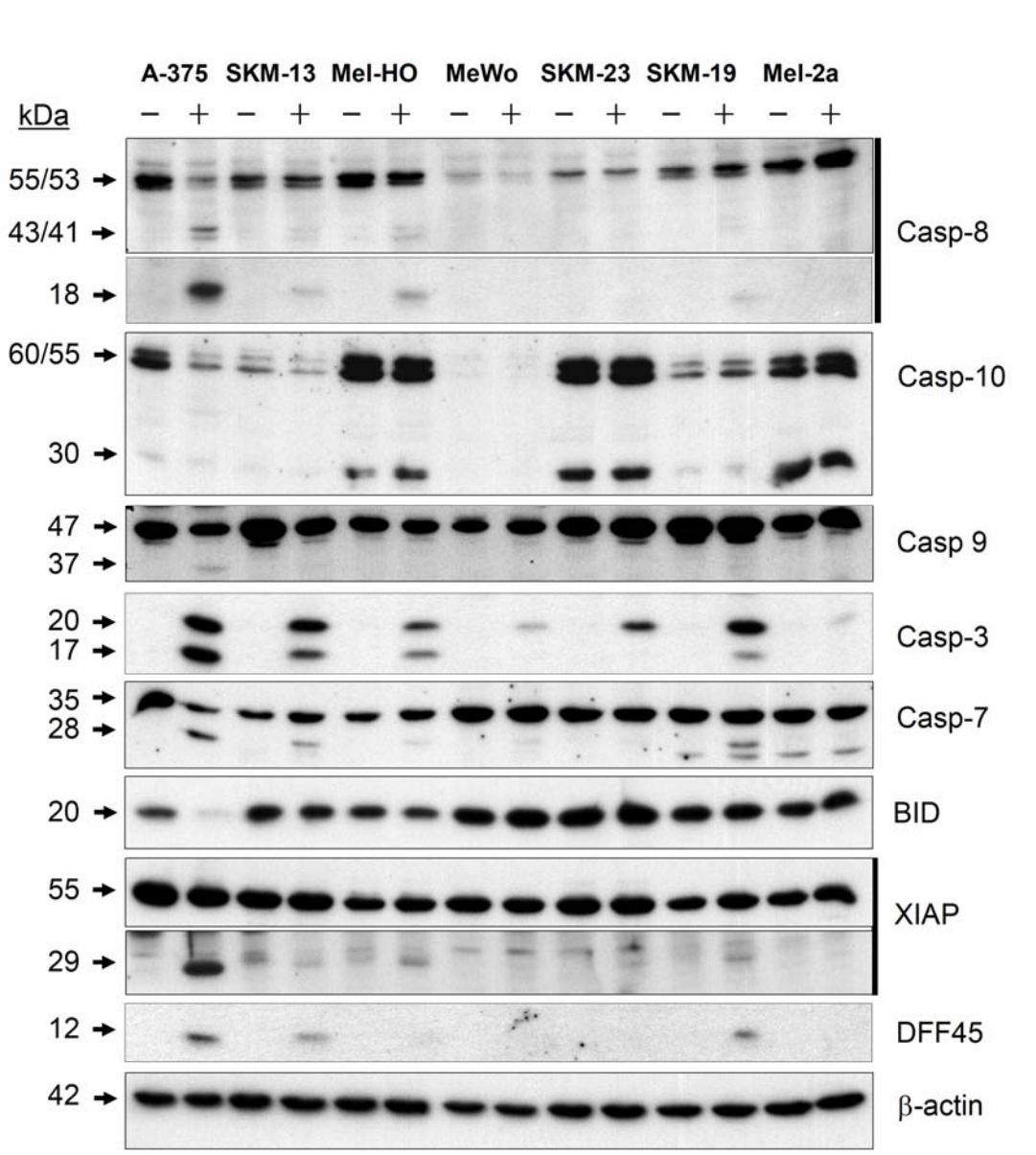

Figure 3.5. Strong caspase activation in DR4-positive melanoma cells

Western blot experiments for capases -8, -10, -9, -3, -7, BID, XIAP and DFF45 are shown for 7 investigated melanoma cell lines. Proteins were extracted from cultures 4 h after TRAIL treatment (+) and from parallel controls (-). For caspase-8 and XIAP, two different exposure times were selected for visualization of the uncleaved and the cleaved forms, respectively. Equal amount of proteins (25 mg per lane) were loaded, and consistent blotting was proven by ponceau staining and by evaluation of β-actin expression. Molecular weights of proforms and cleavage products are indicated. Two experiments performed for each cell line starting from independent cultures revealed highly similar results.

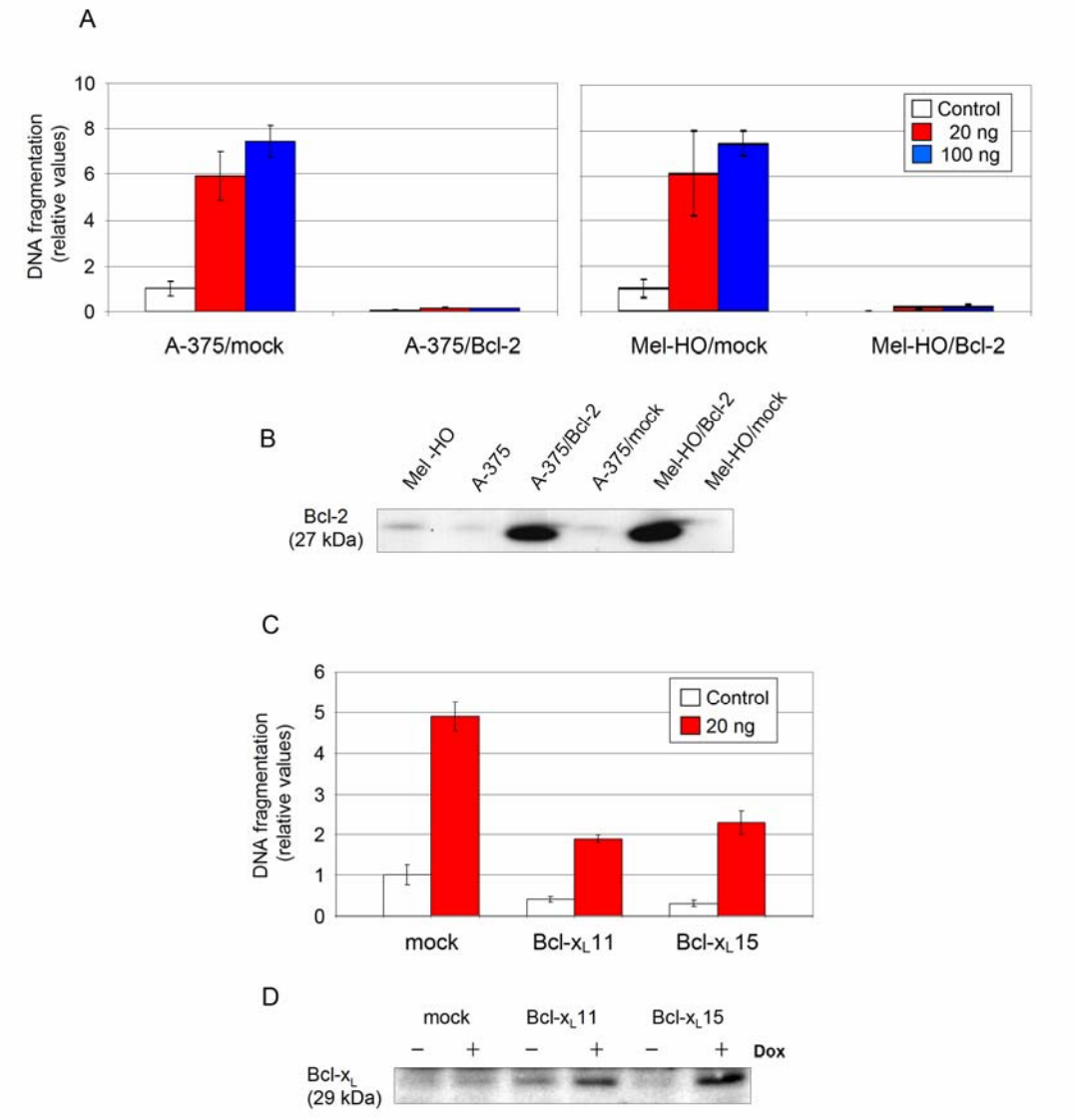

Figure 3.6. TRAIL-induced apoptosis is reduced after overexpression of Bcl-2 and Bcl-x_L

A) Melanoma cell clones stably transfected with Bcl-2 (A-375/Bcl-2 and Mel-HO/Bcl-2) or transfected with vector DNA (A-375/mock and Mel-HO/mock) were treated for 24 h with 20 ng/ml TRAIL (red bars) and 100 ng/ml (blue bars), respectively. Relative values for DNA fragmentation were normalized to the respective untreated mock cells (set to 1). The experiment was performed twice with each time double values, means and standard deviations for all 4 values being given here.
B) Expression of Bcl-2 in mock and in Bcl-2 transfected cells as determined by Western blot analysis.
C) SK-Mel-13 cell clones stably transfected with Bcl-x_L (SKM-13-Bcl-x_{L11} and SKM-13-Bcl-x_{L15}) respectively transfected with vector DNA (SKM-13-mock) were used. Cells were treated as described above for 24 h with 20 ng/ml TRAIL (red bars) or left untreated (white bars). Untreated mock cells were set to 1. Cells were investigated under inducible conditions (2 µg/ml doxycycline). Means and standard deviations of two independent experiments are given, each consisting of triple values (6 values).
D) Expression of Bcl-x_L in mock and in Bcl-x_L-transfected cells as determined by Western blot analysis. Expression is induced in these clones by doxycycline (Dox). Western blot experiments were performed twice resulting in largely identical results.

3.6. Prevalence of DR4 for TRAIL-induced apoptosis in cell lines expressing both receptors

For investigating the respective contribution of both death receptors to TRAIL-mediated apoptosis, cells were treated with monoclonal antibodies blocking selectively DR4 or DR5. Applying DR4 antibodies, apoptosis was largely blocked in A-375 (70%) and in SK-Mel-13 (83%) after 6 h of TRAIL treatment, whereas the effect of DR5 antibodies was less pronounced in these cell lines (23% and 44% inhibition). Almost complete reduction was seen when both antibodies were applied (96%; Fig 3.7.).

Application of DR5 blocking antibodies alone resulted in a significant increase in apoptosis in all melanoma cell lines investigated, most pronounced in Mel-HO and possibly indicative of an agonistic side effect caused by this antibody. Therefore, to calculate of its antagonistic effect, the values were normalized for the agonistic side effect. According to this normalization, DR5 blocking antibodies were able to prevent apoptosis in up to 84% of Mel-HO and in up to 91% in SK-Mel-19. DR4 antibodies remained without any effect in these cell lines (Fig. 3.7.). Cytotoxicity monitored in parallel did not reveal any significant changes in melanoma cells after 6 h (data not shown). These experiments clearly indicate that blocking DR5 worked efficiently but exerted an only minor effect on melanoma cells expressing DR4.

The DR4-positive cell lines A-375 and SK-Mel-13 were further employed in Western blot experiments after applying TRAIL in combination with the two different blocking antibodies. In full agreement with the results from the apoptosis assays, blocking DR4 strongly diminished occurrence of cleavage products for caspase-8, -3 and -7 in A-375 and in SK-Mel-13. Similarly, reduction of Bid as well as occurrence of the 29 kDa XIAP cleavage product and of DFF45 was almost prevented. In contrast, the effects of blocking DR5 were much less pronounced, clearly proving the prevalent role of DR4 for TRAIL-induced apoptosis in these cells (Fig. 3.8.).

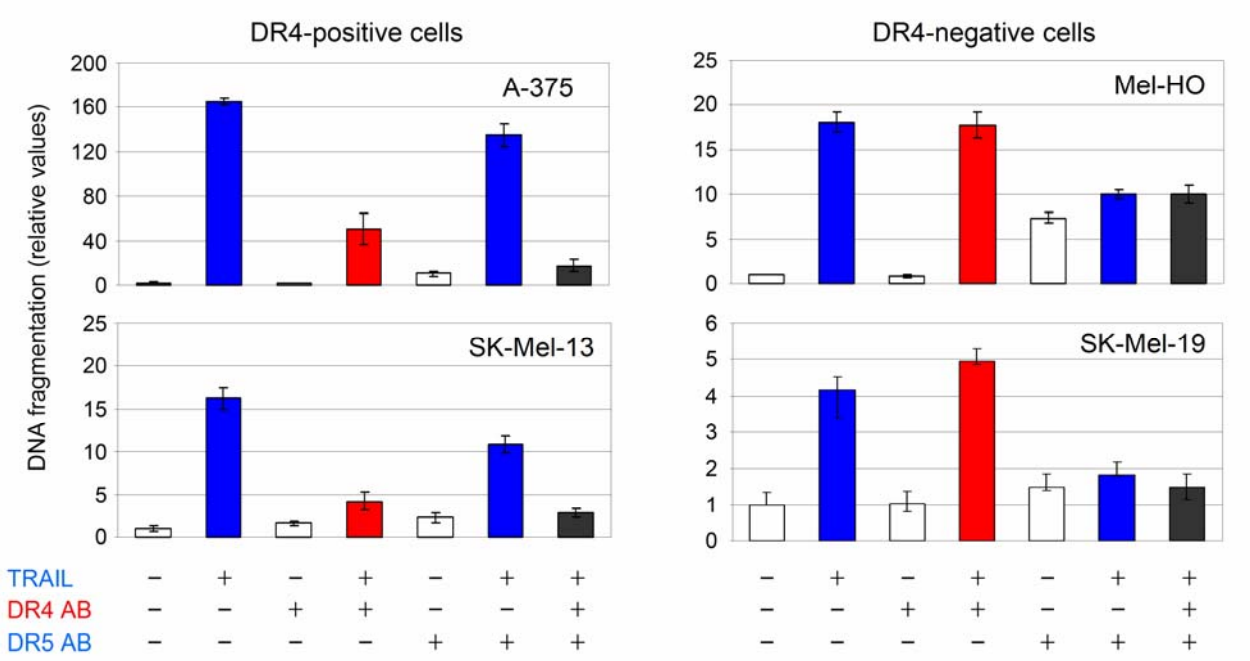

Figure 3.7. Blocking DR4 prevents apoptosis in melanoma cells positive for both receptors

Subconfluent cultures of four melanoma cell lines were pre-incubated for 1 h with blocking antibodies for DR4 and DR5, respectively, before starting treatment with TRAIL (20 ng/ml, for another 6 h). Relative values for DNA fragmentation were calculated with respect to the basic apoptotic rates determined for untreated controls (set to 1, separately for each cell line). Mean values and SD of three independent experiments (A-375, SK-Mel-13) and of two experiments (Mel-HO, SK-Mel-19) are shown, each experiment itself consisted of triple values.

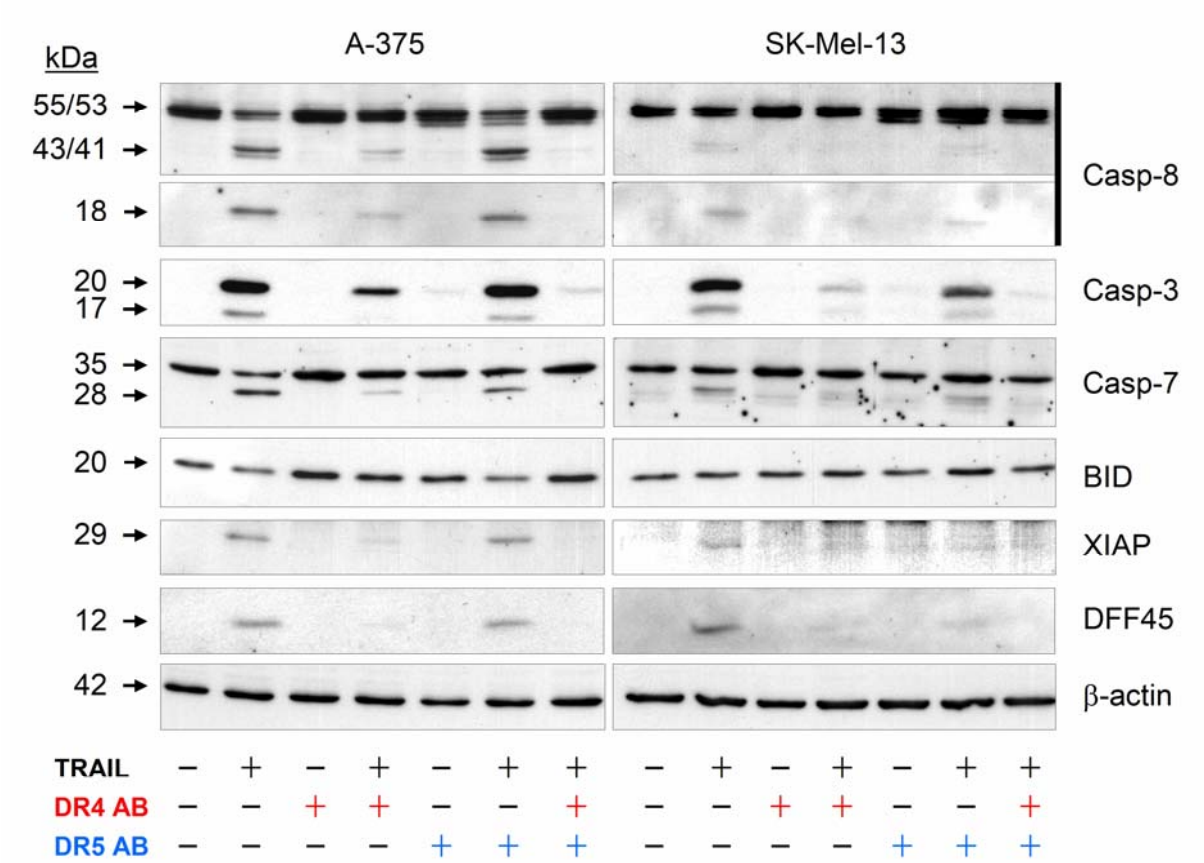

Figure 3.8. Block of apoptotic cascades by DR4 antagonists in A-375 and SK-Mel-13

Western blot analysis for capases -8, -3 and -7 as well as for XIAP and DFF45 are shown for A-375 and SK-Mel-13. Cells were pre-incubated with blocking antibodies for 1 h before starting TRAIL treatment (20 ng/ml, for another 4 h). Equal amounts of proteins (25 mg per lane) were loaded, and consistent blotting was proven by ponceau staining and by evaluation of β-actin expression. The experiment was performed twice starting from independent cultures and revealed highly comparable results.

3.7. Significant expression of DR4 and DR5 in primary melanomas

Expression of DR4 and DR5 was investigated by immunohistochemistry in biopsies of 40 patients with nodular or superficial-spreading melanoma (tumor thickness: 1.5 – 4.0 mm) to show whether DR4 is expressed in primary melanoma and whether the functional significance of DR4, found in this study, could be applied for the in vivo situation. Of the 40 primary tumors examined, 32 (80%) revealed significant expression of both receptors, in 4 tumors (10%) expression for both receptors was weak or almost negative, and in 4 other tumors either DR4 or DR5 was lost (Fig 3.9.).

Specificity of the antibodies applied was proven by immunocytochemistry of melanoma cell lines A-375, SK-Mel-13 and SK-Mel-23. In full agreement with FACS analysis detailed in Fig 3.1, melanoma cell lines A-375 and SK-Mel-13 were stained positively for both DR4 and DR5, whereas SK-Mel-23 was negative for DR4 but highly positive for DR5 (Fig 3.9.). These data clearly indicate that expression of DR4 was as frequent as of DR5 in melanoma, which is possibly indicative of the significance of both receptors in melanoma patients.

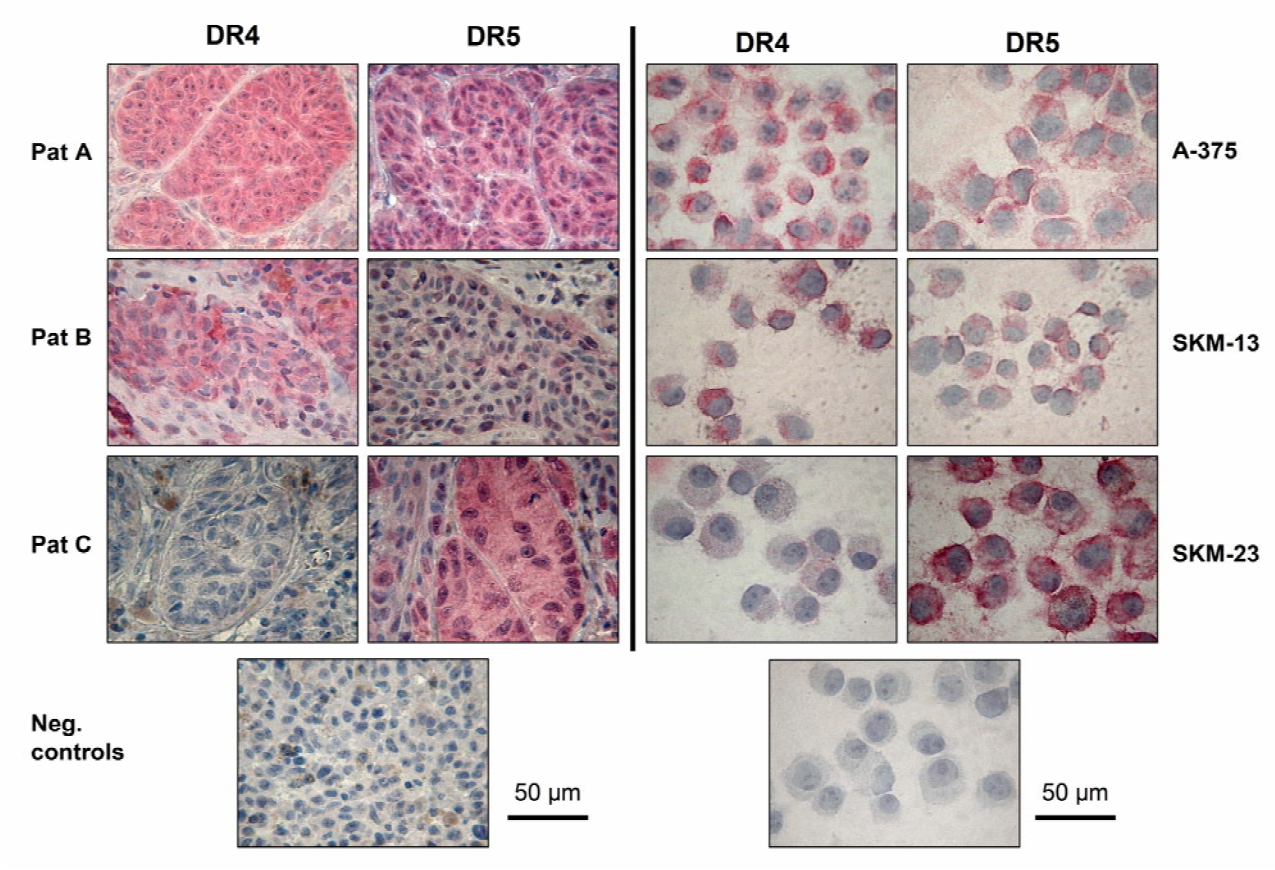

Figure 3.9. Significant expression of both TRAIL death receptors in melanoma biopsies

The expression level of DR4 and DR5 was examined by immunohistochemistry in primary tumors from 40 patients with nodular or superficial-spreading melanoma. Three examples are shown (left side) with strong expression of both receptors (patient A), strong expression of DR4 and weak expression of DR5 (patient B) as well as with missing expression of DR4 and strong expression of DR5 (patient C). Immunocytochemistry for melanoma cell lines A-375, SK-Mel-13 and SK-Mel-23 shown on the right side proved specificity of the antibodies applied and demonstrated a comparable situation in vitro and in vivo with respect to receptor expression. Negative controls for immunohistochemistry and for immunocytochemistry (no primary antibody) as well as magnification scales are indicated below.

3.8. DR4-positive melanoma cells reveal high NF-κB activation after TRAIL treatment

For subsequent investigations, five melanoma cell lines and cultures of normal human melanocytes (NHM) were applied. According to our previous data, the cell lines were subdivided in sensitive (A-375, SK-Mel-13, Mel-HO) and resistant (SK-Mel-23, MeWo and NHM) to TRAIL-induced apoptosis. Two cell lines were positive for DR4 and DR5, whereas Mel-HO, SK-Mel-23, MeWo and NHM only expressed DR5.

To determine whether TRAIL may induce nuclear translocation and activation of NF-κB, four melanoma cell lines (A-375, SK-Mel-13, Mel-HO; SK-Mel-23) and two NHM cultures were investigated by an ELISA assay specific for the five subunits of NF-κB (p65, p50, c-Rel, Rel-B and p52). Levels of c-Rel, Rel-B and p52 remained unchanged upon TRAIL treatment (100 ng/ml, 1h) in all cell lines. Nuclear translocation was however seen for p65 and p50 in the DR4-positive cell lines A-375 and SK-Mel-13 ($p < 0.05$), whereas there was no significant change in NF-κB binding activities in cells lacking DR4 (Fig 3.10.a).

A highly comparable activation pattern was obtained investigating the cells by EMSA analysis. Interestingly, the TRAIL-sensitive Mel-HO showed the highest constitutive nuclear binding activities, whereas the TRAIL-resistant SK-Mel-23 did not reveal significant activities. Identity of NF-κB subunits in the complexes was proven by supershift analysis, which was highly indicative of p65/p50 dimers (Fig 3.11.). Thus, NF-κB activation by TRAIL was restricted to DR4-positive melanoma cells.

To find out whether NF-κB activation was completely blocked in DR4-negative cells, its binding activity was also determined in Mel-HO and A-375 after treatment with TNF-α (10 ng/ml, 1h). Unlike TRAIL, TNF-α was able significantly to increase p65 NF-κB nuclear binding activity in Mel-HO ($p < 0.05$; Fig 3.12.).

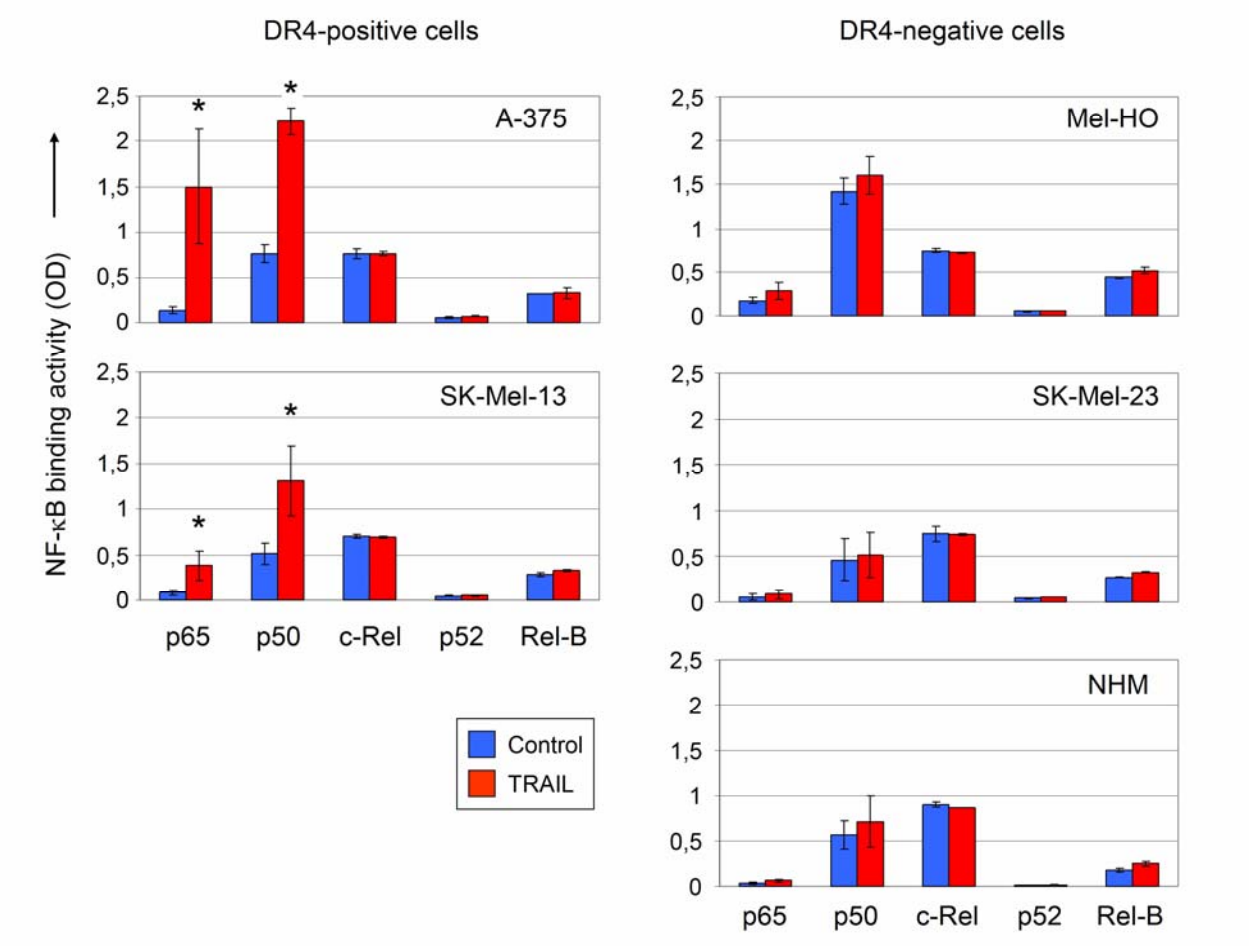

Figure 3.10. TRAIL triggers NF-κB activation only in DR4-expressing melanoma cells

(A) DR4-positive melanoma cell lines (A-375, SK-Mel-13) as well as DR4-negative cell lines (Mel-HO, SK-Mel-23) and normal human melanocytes (NHM) were treated with 100 ng/ml TRAIL (red) and compared with controls (blue). Nuclear extracts were prepared 1 h after treatment, and equal amounts of nuclear proteins (7 µg) were analyzed for binding activities of the five NF-κB subunits (p65, p50, c-Rel, p52, Rel-B) by ELISA analysis. Data reflect mean values and SD (OD; three independent experiments). Significant increases are indicated by asterisks.

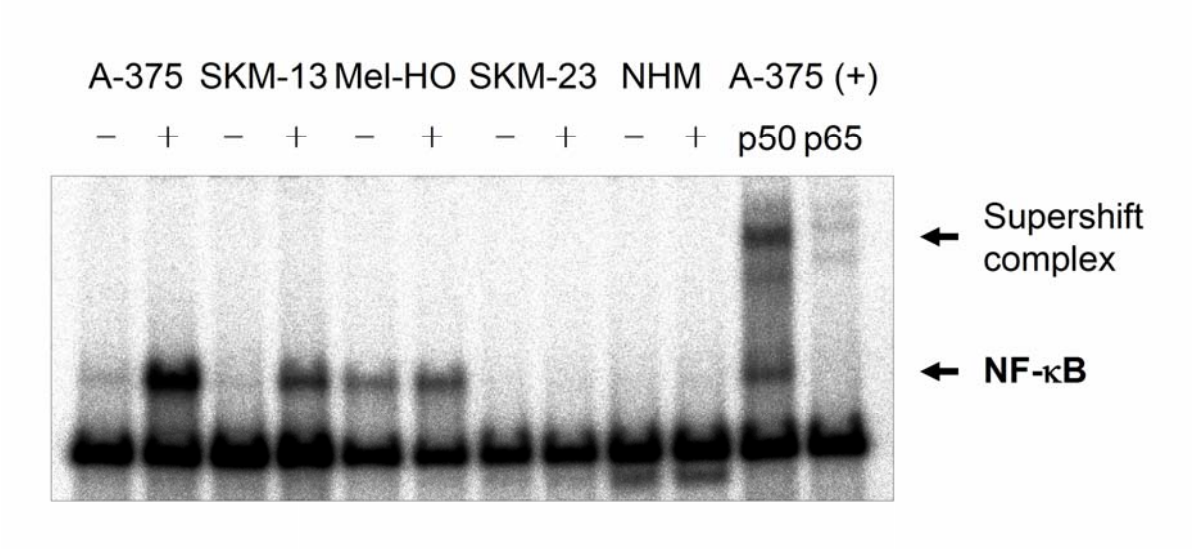

Figure 3.11. High NF-κB nuclear binding activity in DR4-positive melanoma cells by EMSA

Nuclear extracts were further submitted to EMSA analysis. Equal amounts of nuclear proteins (7 µg) were used. Cultures treated with TRAIL (+) were compared to untreated cells (-). The shifted bands, which characterize NF-κB binding activity, are indicated (NF-κB). Supershift complexes are shown on the left for TRAIL-treated A-375 melanoma cells after incubation with specific p65 and p50 antibodies. Two independent experiments revealed highly comparable results.

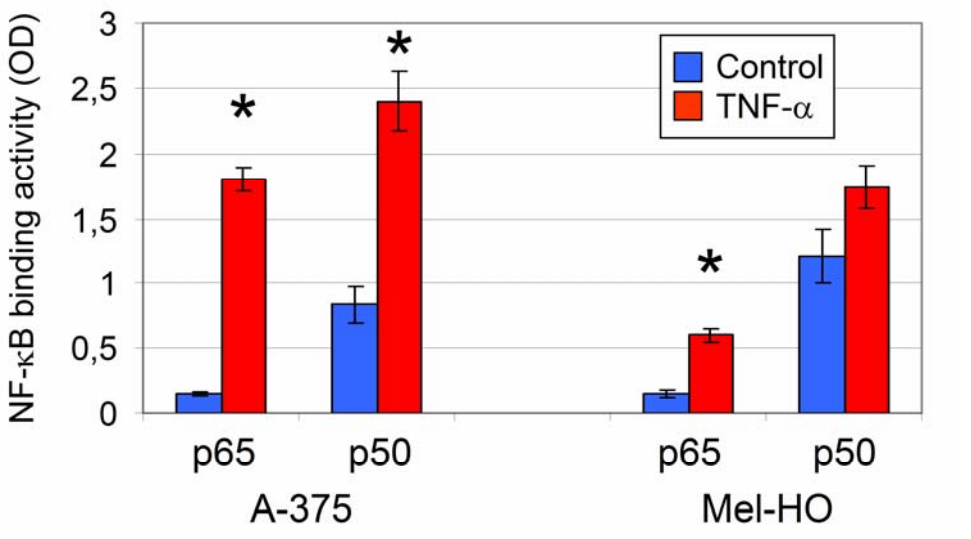

Figure 3.12. NF-κB is activated by TNF-α in DR4-negative melanoma cells

Melanoma cell lines A-375 and Mel-HO were treated with 10 ng/ml TNF-α (red) and were compared with controls (blue). After 1 h, nuclear binding activities of p65 and p50 of NF-κB subunits were determined by ELISA. Equal amounts of nuclear proteins (7 µg) were used. Data reflect mean values of the binding activities (OD; three independent cultures of each cell line). Significant increases are indicated by asterisks.

3.9. Parallel apoptosis induction and NF-κB activation by TRAIL in DR4-positive melanoma cells

Dose-response curves for A-375 as well as kinetic analyses for A-375 and Mel-HO were established for the binding activities of p65 and p50 as well as for the apoptotic response. A significant dose-dependent increase in NF-κB nuclear binding activities was seen in A-375 for both NF-κB subunits, starting with 20 ng/ml TRAIL. Apoptosis, which was determined after 6 hours, already started to increase in A-375 at TRAIL concentrations above 1 ng/ml (Fig 3.13.a). The kinetic analysis of NF-κB binding activity revealed an increase for both subunits as early as 1 h after TRAIL stimulation (100 ng/ml), and it reached its peak after 2 h in A-375. Apoptosis induction closely followed NF-κB activation starting at 1 h after TRAIL stimulation (Fig 3.13.d).

These results show that TRAIL signalling led to apoptosis even at low concentrations, whereas NF-κB activation required higher concentration of ligand. Furthermore, apoptosis and NF-κB activation after TRAIL stimulation were largely in parallel in DR4-positive A-375 cells, whereas there was no NF-κB activation in DR4-negative Mel-HO over the whole period of observation (12 h).

3.10. Increased NF-κB activity by TRAIL is mediated thought DR4

For determining which death receptor triggers NF-κB activation in cell lines positive for both TRAIL receptors, blocking antibodies were applied, which specifically bind and inactivate either DR4 or DR5. The selective activity of these antibodies had been proven in previous investigations, where DR4 antagonistic antibodies abolished TRAIL-induced apoptosis in DR4-positive cells, whereas DR5 antibodies abolished apoptosis in DR4-negative (DR5-positive) cells (see Figure 3.7.). Here, blocking of DR4 prevented almost completely the nuclear binding activities of p65 and p50 subunits of NF-κB in A-375 after TRAIL treatment (100 ng/ml), while there was no blocking effect of DR5 antibodies (Fig 3.14.).

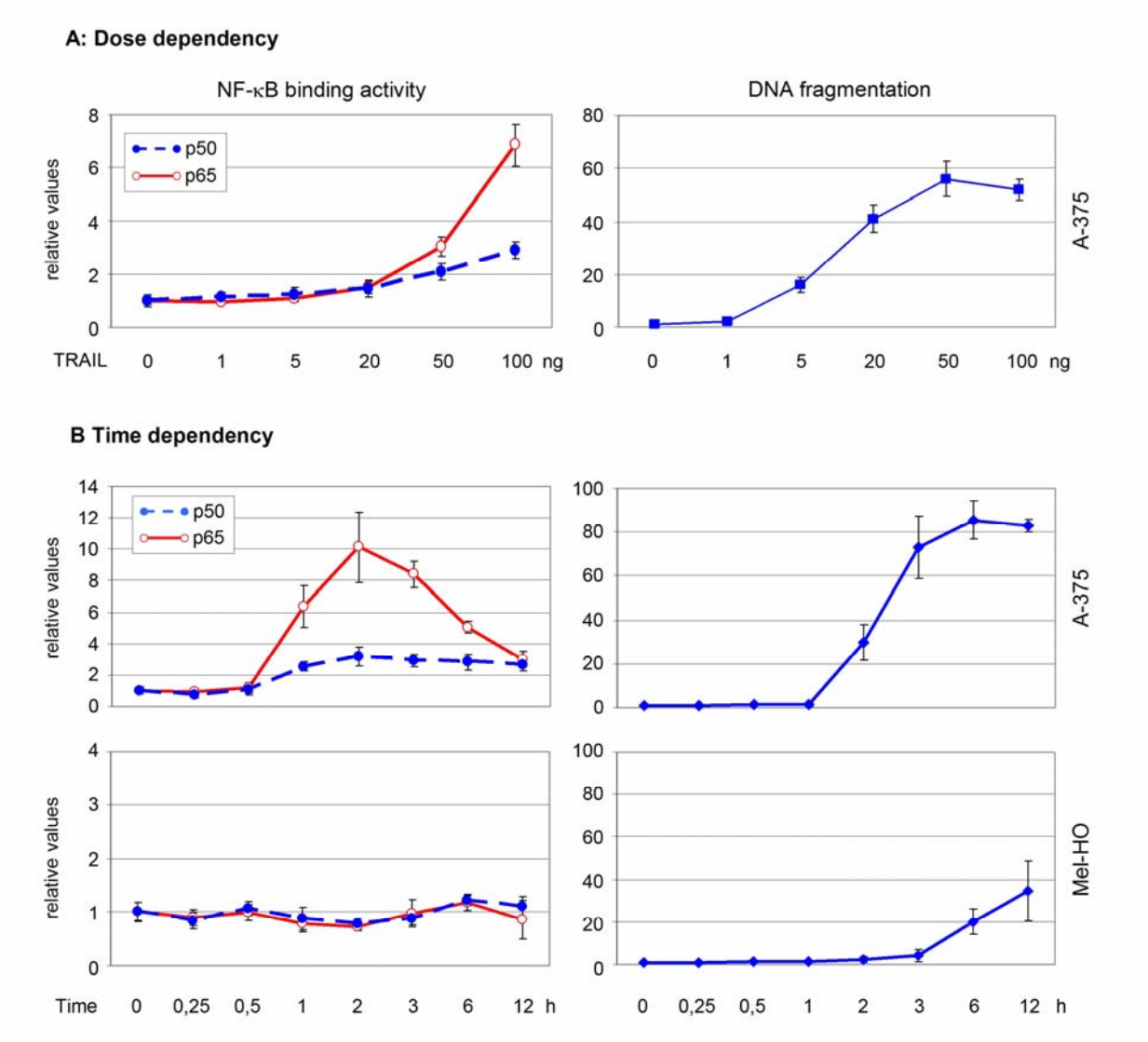

Figure 3.13. Dose and time dependence of NF-κB activation and apoptosis in melanoma cells

(A) Dose dependency. A-375 cells were treated with different concentrations of TRAIL as indicated, and nuclear extracts were prepared after one hour. Equal amounts of nuclear proteins were analyzed for binding activities of NF-κB subunits (p65 and p50) by ELISA. DNA fragmentation rates (below) were determined in parallel cultures treated for 6 hours with TRAIL. Mean values and SD of two independent experiments are shown.
(B) Time dependency. A-375 and Mel-HO were stimulated with TRAIL (100 ng/ml) for times as indicated. NF-κB binding activity (p65, p50) and DNA fragmentation were determined from parallel cultures. Mean values and SD of three independent experiments are shown.

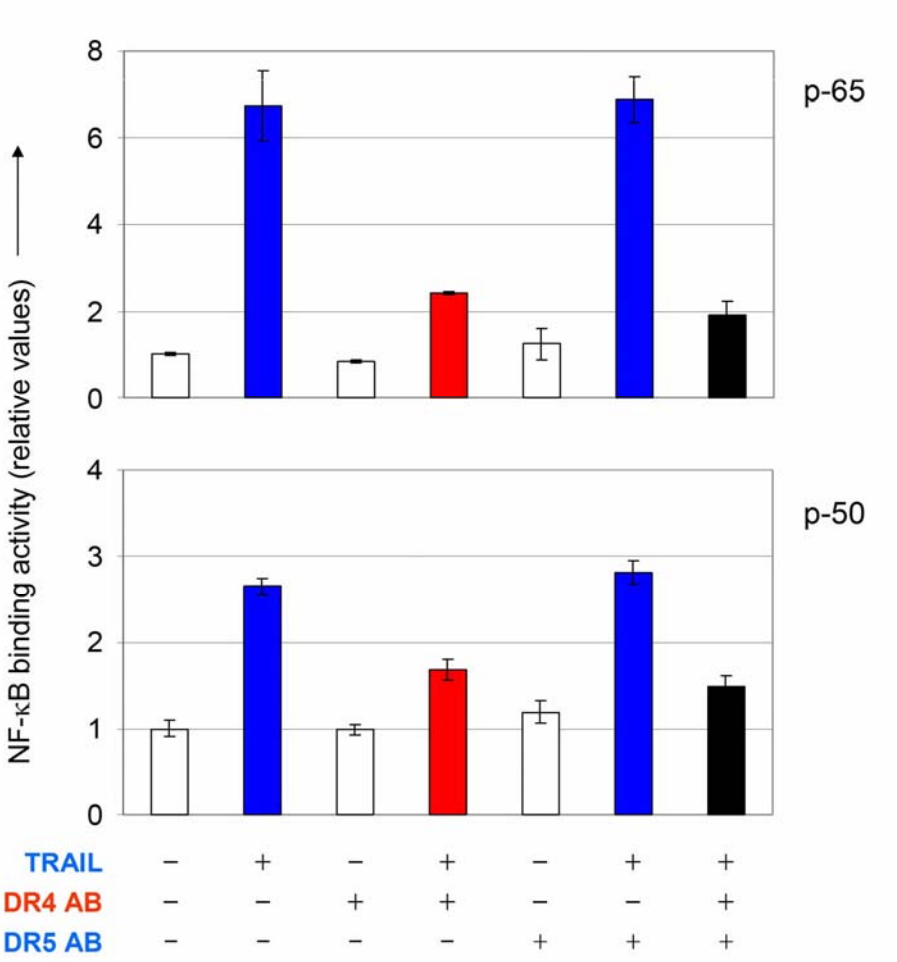

Figure 3.14. High NF-κB binding activity upon TRAIL treatment is mediated by DR4

A-375 melanoma cells were pre-incubated for 1 h with blocking antibodies specific for DR4 and for DR5, respectively, before cells were further treated with TRAIL (100 ng/ml, for another 1 h). Relative values of NF-κB nuclear binding activities were calculated with respect to untreated controls, which were set to 1. Mean values and SD of three independent experiments are shown.

3.11. No changes in the levels of anti-apoptotic proteins after TRAIL treatment but decrease of DR4 in SK-Mel-13

We further investigated whether activation of NF-κB via DR4 may trigger expression of anti-apoptotic proteins. DR4 positive (A-375, SK-MEL-13) and negative cells (Mel-HO) were treated for 24h with TRAIL and TNF-α, respectively. Western blot analyses for antiapoptotic proteins (Survivin, Bcl-x_L, XIAP, Livin, Bcl-2 and c-FLIP) did not reveal significant changes after treatment despite some tendency for increased Survivin in A-375 and Mel-HO (Fig

3.15.). However, there was a significant downregulation found for DR4 in SK-Mel-13 cells after TRAIL treatment, no changes were seen for DR5, nor for the other cell lines investigated (Fig 3.16.a).

To determine whether downregulation of DR4 resulted from NF-κB activation, proteasome inhibition experiments were applied. The proteasome inhibitor (LLnL) is known to prevent IκB degradation, which in turn leads to a blocking of NF-κB activation. Interestingly, the TRAIL-induced downregulation of DR4 was completely prevented by pre-treatment for 2 h with LLnL as shown in a kinetic study (Fig 3.16.b). The inhibition effect of LLnL on activation of NF-κB has been shown in separate experiments where LLnL could prevent TRAIL-induced NF-κB activation in SK-Mel-13 cells (data not shown).

With respect to cell survival, pre-treatment with LLnL caused a strong increase of the killing effect of TRAIL in all melanoma cell lines investigated, including Mel-HO and SK-Mel-23, in which TRAIL did not induce NF-κB activity (Fig 3.17.). The synergism found for combined treatment of TRAIL and LLnL seems therefore not exclusively related to a blocking of NF-κB activation by LLnL. For SK-Mel-13, prevention of DR4 downregulation may contribute to its increased sensitivity after LLnL treatment.

3.12. Transient resistance of DR4 melanoma cells selected with TRAIL

To determine whether TRAIL-induced NF-κB activation exerts its antiapoptotic features in resistant cells, a selection of TRAIL resistant melanoma cells was performed. TRAIL-resistant cell populations were selected from the three TRAIL-sensitive cell lines A-375, SK-Mel-13 and Mel-HO by continuous culturing these cells in the presence of TRAIL for 8-10 weeks. Selected cell populations could be cultured at TRAIL concentrations of up to 100 ng/ml. Starting from these resistant populations, recovery of sensitivity was monitored 1 and 3 months after TRAIL withdrawal from culture medium. Whereas selected cell populations were largely resistant to TRAIL-induced apoptosis, DR4-positive cell lines recovered sensitivity after 1 month (A-375) or after 3 months (SK-Mel-13). In contrast, the DR4-negative, TRAIL-selected Mel-HO maintained resistance even 3 months after TRAIL withdrawal (Fig 3.18.).

Selected, TRAIL-resistant melanoma cells also revealed cross-resistance to the agonistic CD95 antibody CH-11 as well as to TNF-α, and sensitivity to these agonists was also partly recovered after TRAIL withdrawal (Fig 3.19.), indicating that mechanism of resistance also affected general pathways common to all death receptors.

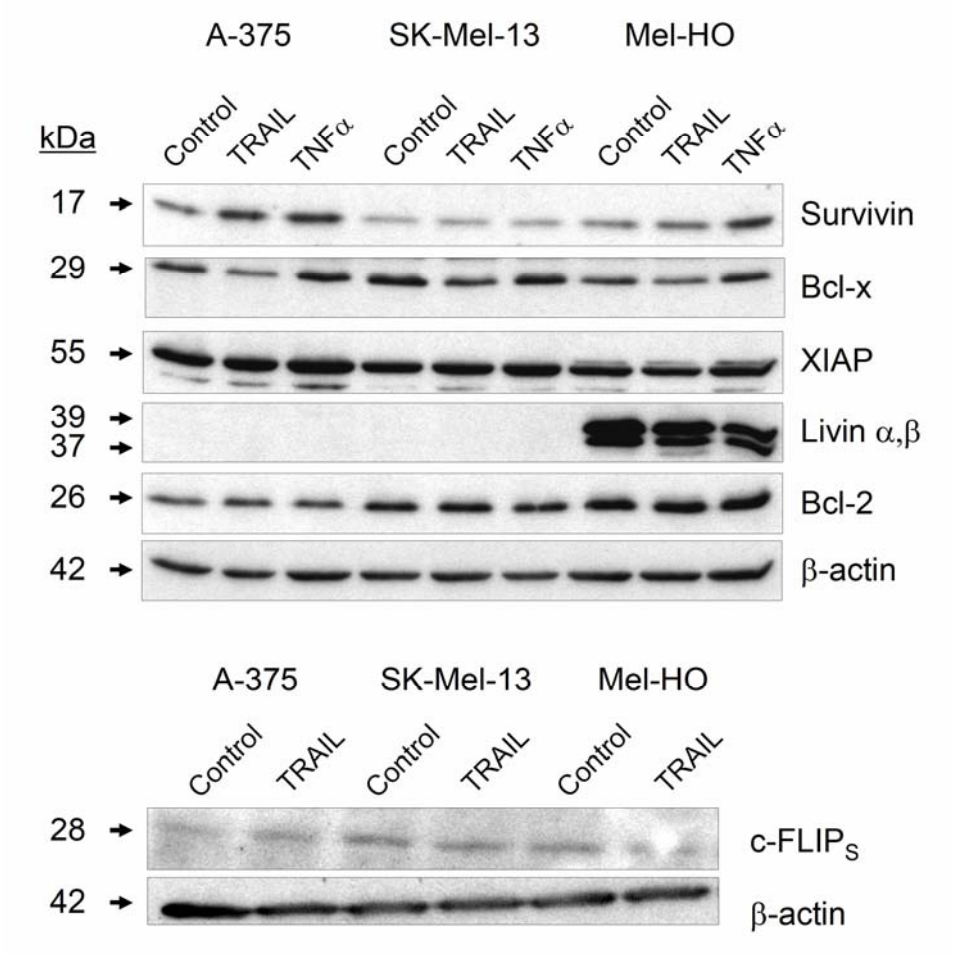

Figure 3.15. No effect of TRAIL-induced NF-κB on expression of antiapoptotic proteins
Western blot analyses of a series of antiapoptotic proteins (Survivin, Bcl-x_L, XIAP, FLIPs, Livin and Bcl-2) are shown for three melanoma cell lines. Proteins were extracted 24 h after treatment with TRAIL (100 ng/ml) or TNF-α (10 ng/ml). They were compared to untreated controls. Equal protein amounts (40 µg per lane) were loaded, and consistent blotting was proven by ponceau staining and by evaluation of β-actin expression shown below. Molecular weights are indicated on the left. Two experiments for each cell line starting from independent cultures revealed highly similar results.

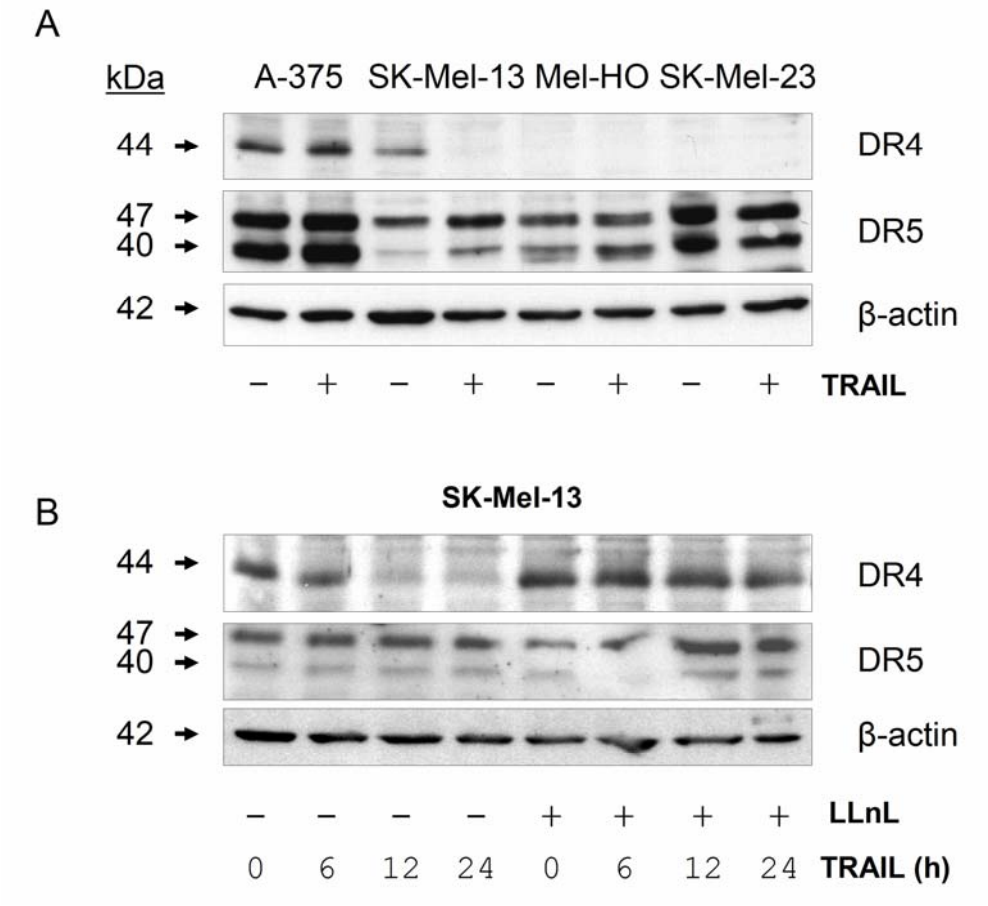

Figure 3.16. Downregulation of DR4 by TRAIL and its prevention by LLnL
(A) Four melanoma cell lines were treated with 100 ng/ml TRAIL (+) and were compared to untreated control (-). Protein extracts were prepared after 24 hours, and equal protein amounts (40 µg per lane) were loaded for determination of DR4 and DR5 expression by Western blot analysis. Three experiments for each cell line starting from independent cultures revealed highly similar results, namely downregulation of DR4 by TRAIL in SK-Mel-13.
(B) SK-Mel-13 cells were pre-treated with the proteasome inhibitor LLnL (5 µM), 2 h before starting TRAIL treatment (100 ng/ml). The time dependency of DR4 and DR5 levels was determined by Western blots analysis 0, 6, 12 and 24 h after starting TRAIL treatment in LLnL pre-treated cells (+) and cells that did not received LLnL (-). The proteasome inhibitor was present throughout the experiment. Two experiments for each cell line starting from independent cultures revealed highly similar results.

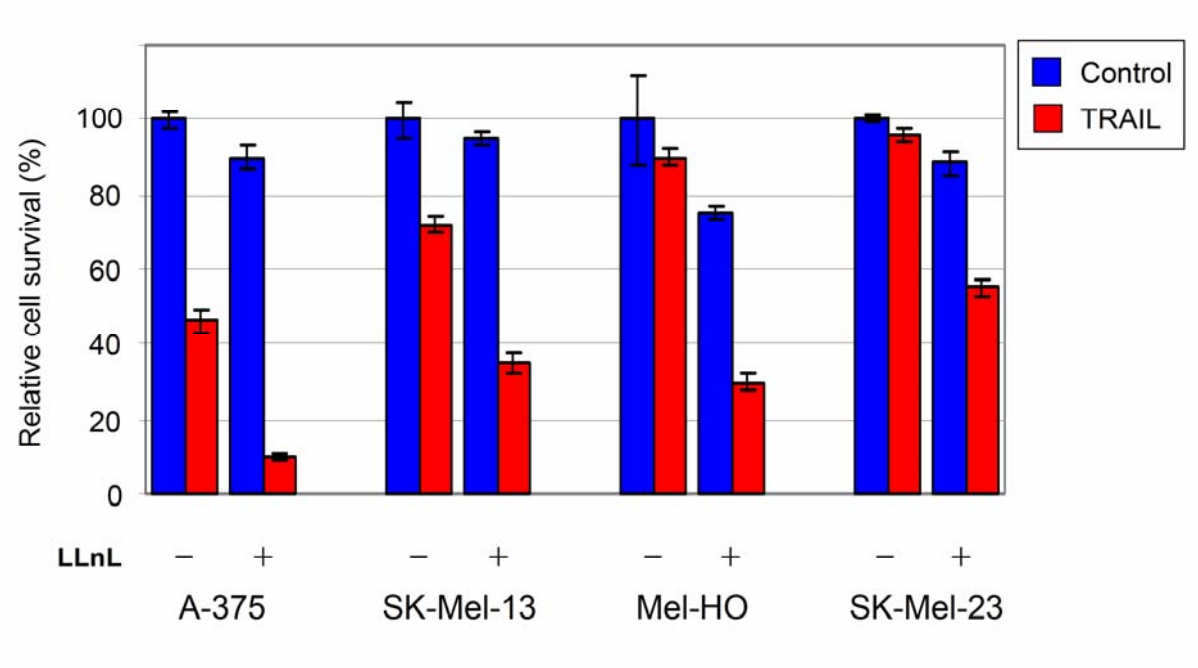

Figure 3.17. Proteasome inhibitor LLnL enhances TRAIL sensitivity independently of NF-κB activation

Four melanoma cell lines were pre-treated with the proteasome inhibitor LLnL (5 μM), 2 h before starting TRAIL treatment (100 ng/ml, for 24 h). Survived cells were subjected to a crystal violet viability assay. Relative cell numbers were normalized to untreated controls, which were set to 100% for each cell line. Means and SD of two independent experiments performed in triplicate are presented.

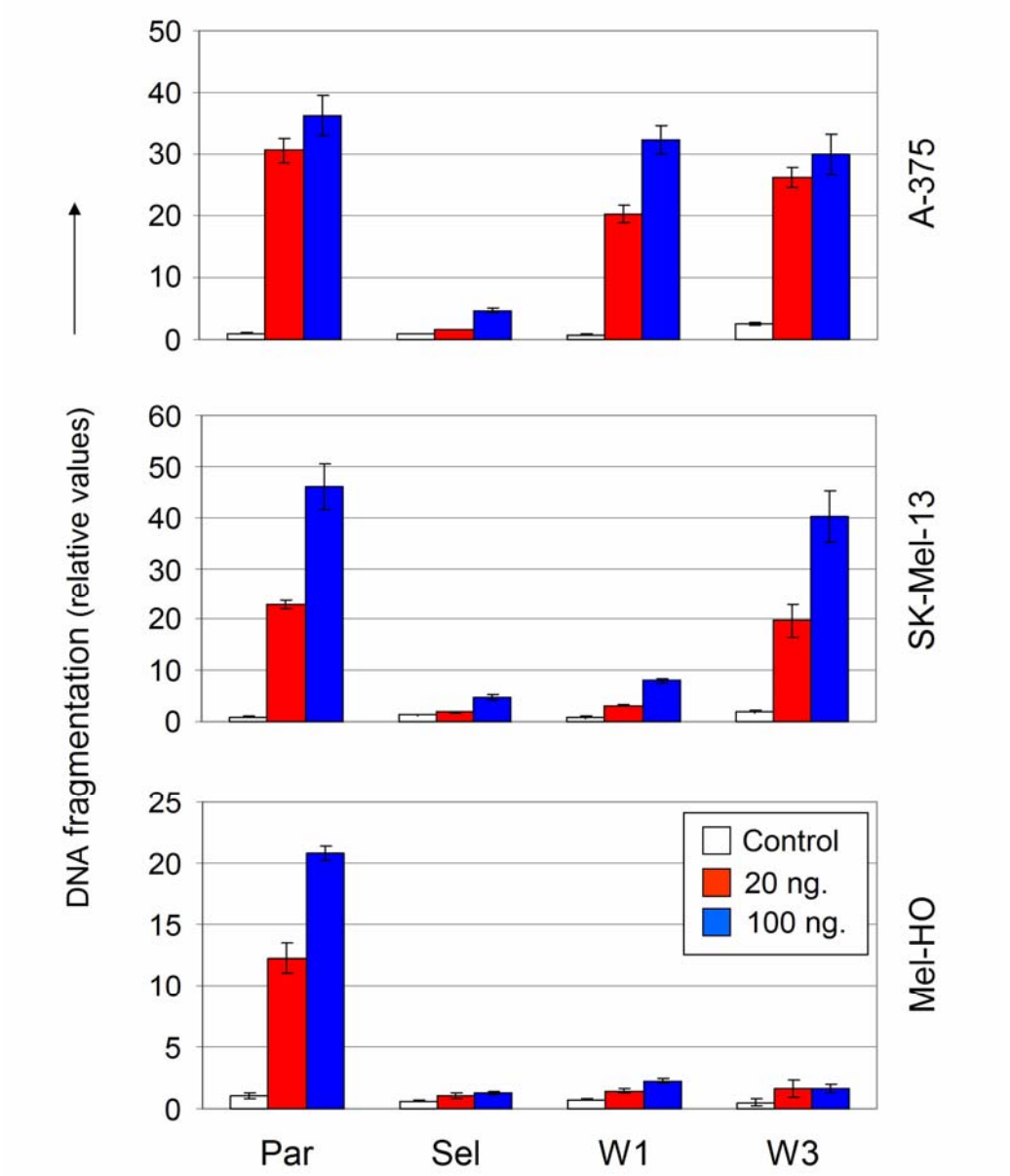

Figure 3.18. DR4-positive, TRAIL selected melanoma cells showed transient resistance to TRAIL

Three parent melanoma cell lines (Par) were investigated: A-375, SK-Mel-13 and Mel-HO. TRAIL-resistant subcultures (Sel) were selected by continuous cultivation for 8-10 weeks in presence of TRAIL. Recovery of TRAIL sensitivity was monitored 1 month (W1) and 3 months after withdrawal of TRAIL (W3). DNA fragmentation rates were determined 6 h after starting treatment with TRAIL (20 ng/ml and 100 ng/ml) and were compared to untreated controls. The controls of the parent cells were set to 1 for each cell line. Mean values and SD of three independent experiments each performed in duplicates are shown.

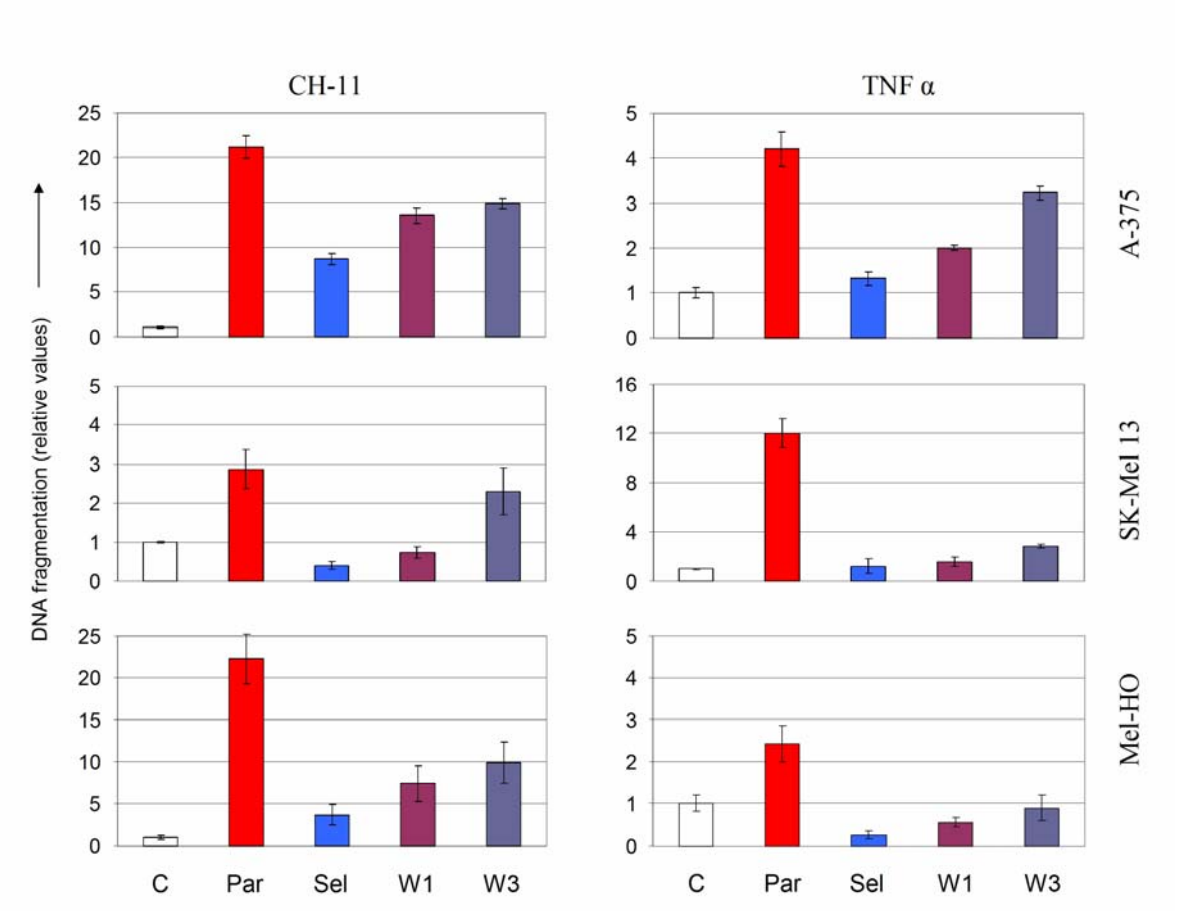

Figure 3.19. TRAIL-resistant selected melanoma cell populations show cross-resistance to CH-11 and TNF-α

The cells mentioned above (Par, Sel, W1, W3) were treated with CH-11 (500 ng/ml) or with TNF-α (10 ng/ml). DNA fragmentation rates were determined 24 h after ligand treatment. Untreated parent cell lines (C) were set to 1. Mean values and SD of two independent experiments each performed with triple values are shown.

3.13. Reduced activation of NF-κB in TRAIL-resistant melanoma cells

To find out, whether high NF-κB activation can be measured in selected melanoma cells, nuclear binding activities of p65 and p50 were determined after TRAIL and after TNF-α treatment in A-375, SK-Mel-13 and Mel-HO parent and selected cell cultures. TRAIL-induced NF-κB activation was almost completely blocked in selected SK-Mel-13 cells, and it was reduced in A-375 selected cells. TRAIL-induced NF-κB activation was not found in selected Mel-HO cells. In contrast, NF-κB activation by TNF-α was not diminished in

selected cells (Fig 3.20.). Thus, these findings clearly exclude, that acquired TRAIL-resistance in melanoma cells is the result of NF-κB activation.

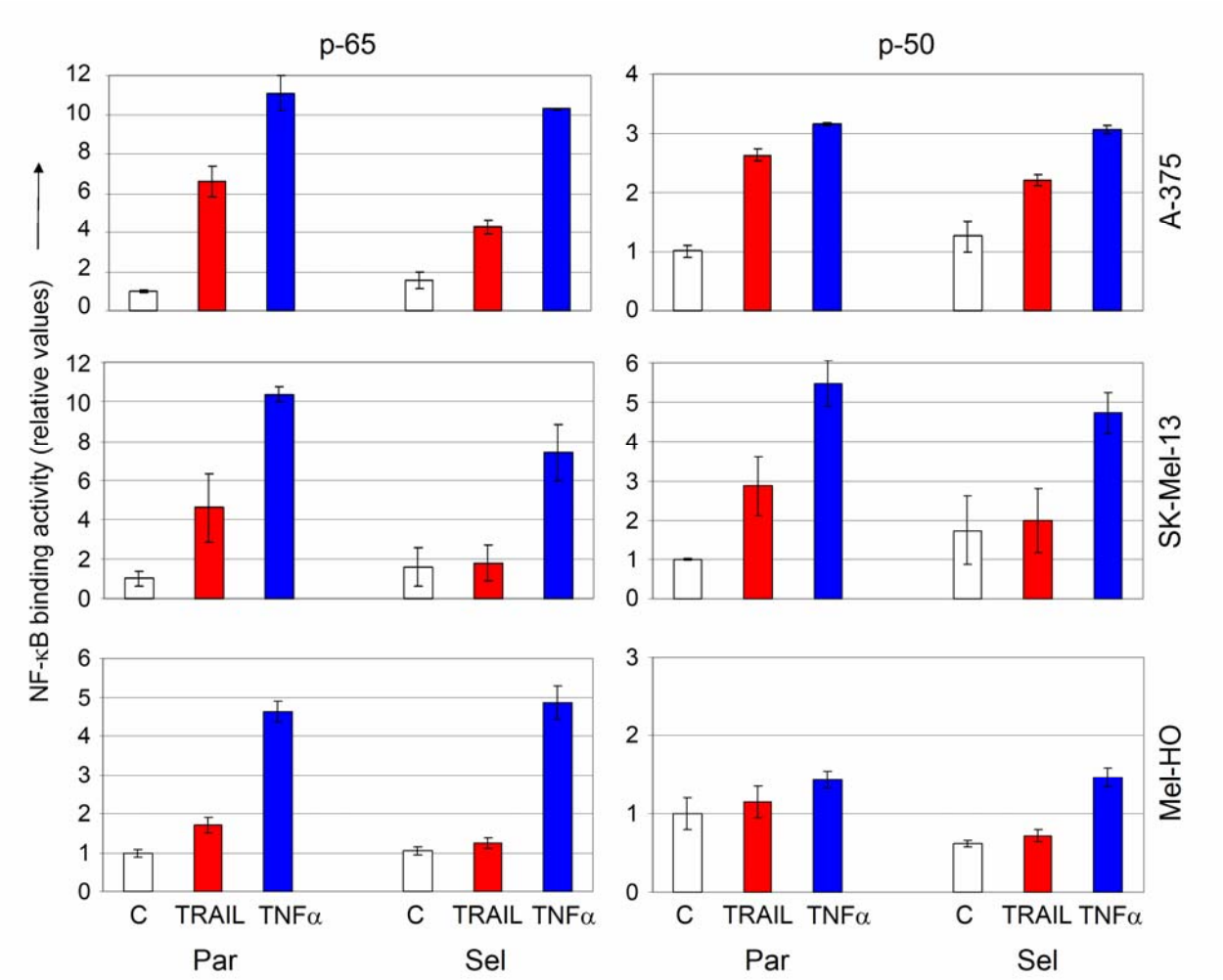

Figure 3.20. NF-κB activation is reduced in TRAIL-resistant melanoma cells

Parent and selected melanoma cell lines (A-375, SK-Mel-13, Mel-HO) were treated with TRAIL (100 ng/ml) or with TNF-α (10 ng/ml) as indicated below. Nuclear extracts were prepared one hour after starting treatment. Equal amounts of nuclear proteins (7 µg) were analyzed for binding activities of NF-κB subunits p65 and p50 by ELISA. Data reflect relative values of the binding activities as compared to untreated controls (means and SD of three independent cultures of each cell line).

3.14. TRAIL resistance of melanoma cells is related to downregulation of initiator caspases and DR4

For understanding the nature of TRAIL resistance and the recovery of sensitivity in melanoma cells, we investigated the expression of a panel of anti- and proapoptotic factors by Western blot analysis in parent cells, selected cells and cells collected 1 month after TRAIL withdrawal. No change of expression was seen for the antiapoptotic proteins Survivin, XIAP, Livin, Bcl-2, c-FLIP and Bcl-x_L as well as for the proapoptotic factors DR5, caspase-3, caspase-7 and Bax. However, strong downregulation of both initiator caspases of the extrinsic pathway (8 and 10) was found in all three selected, TRAIL-resistant cultures, and their expression was largely recovered 1 month after TRAIL withdrawal. In addition there was a strong decrease in DR4 exclusively in SK-Mel-13 and a decrease in Bid in Mel-HO selected cells, which were also recovered after 1 month (Fig 3.21.). Thus, TRAIL resistance in melanoma seemed to be more dependent on downregulation of proapoptotic factors rather than on NF-κB-mediated upregulation of antiapoptotic factors.

3.15. TRAIL sensitivity can be restored by overexpression of initiator caspases and DR4 in selected cells and in resistant MeWo cells

For proving the significance of downregulated initiator caspases and DR4, selected A-375 and SK-Mel-13 cells were transiently transfected with expression plasmids encoding caspase-8, caspase-10, DR4 or DR5. Due to insufficient transfection efficiency, Mel-HO was not included in these experiments. Instead, we employed the parent melanoma cell line MeWo, which we characterized as TRAIL-resistant, DR4-negative, DR5-positive and with weak expression of initiator caspases.

Overexpression of caspase-8 as well as of caspase-10 after transient transfection was able largely to restore TRAIL sensitivity in A-375 and in SK-Mel-13 selected cells as well as in resistant MeWo cells (Fig 3.22.a). Some increase in basic apoptosis found after caspase-8 transfection might be the result of its spontaneous cleavage seen as 41 kDa and 43 kDa cleavage products. Overexpression was confirmed by Western blot analysis (Fig 3.22.b). In addition, typical morphological changes of nuclei, characteristic for apoptosis (nuclear condensation and fragmentation) were observed in the A-375-selected melanoma cells after overexpression of caspase-8 by labeling with Hoechst-33258 (Fig. 3.23.).

Similarly, overexpression of DR4 significantly increased TRAIL sensitivity in these cells. In clear contrast, massive overexpression of DR5 remained without effect on TRAIL sensitivity (Fig 3.24.a). Overexpression was confirmed by Western blot analysis (Fig 3.24.b).
Thus, downregulation of both initiator caspases and of DR4 turned out as critical factors leading to TRAIL resistance in melanoma cells.

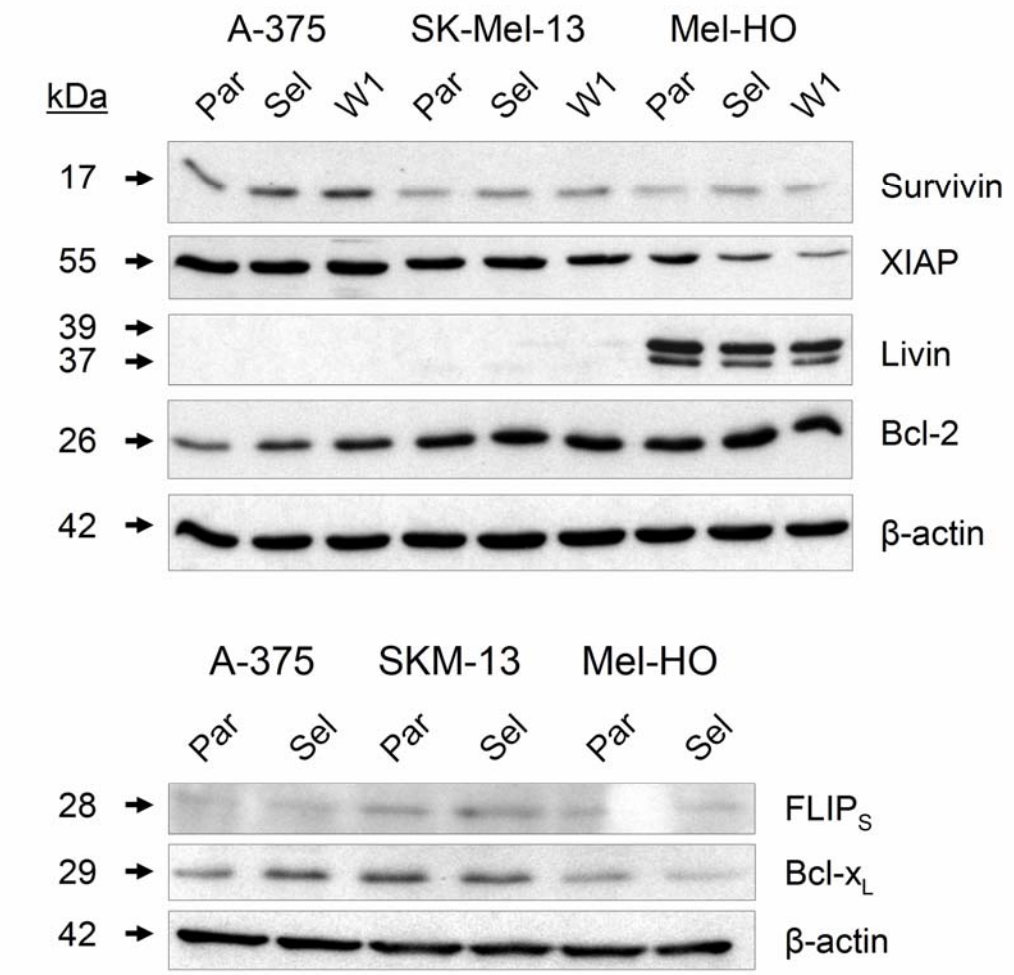

Figure 3.21. No changes in expression of antiapoptotic protein in selected, TRAIL-resistant melanoma cells

The expression of antiapoptotic proteins as Survivin, XIAP, $FLIP_S$, Livin, Bcl-x_L and Bcl-2 were investigated by Western blot analysis in parent cells (Par), TRAIL-resistant subcultures (Sel) and cultures collected one month after TRAIL withdrawal (W1). Equal protein amounts (40 µg per lane) were loaded. Two independent experiments for each cell line revealed highly similar results.

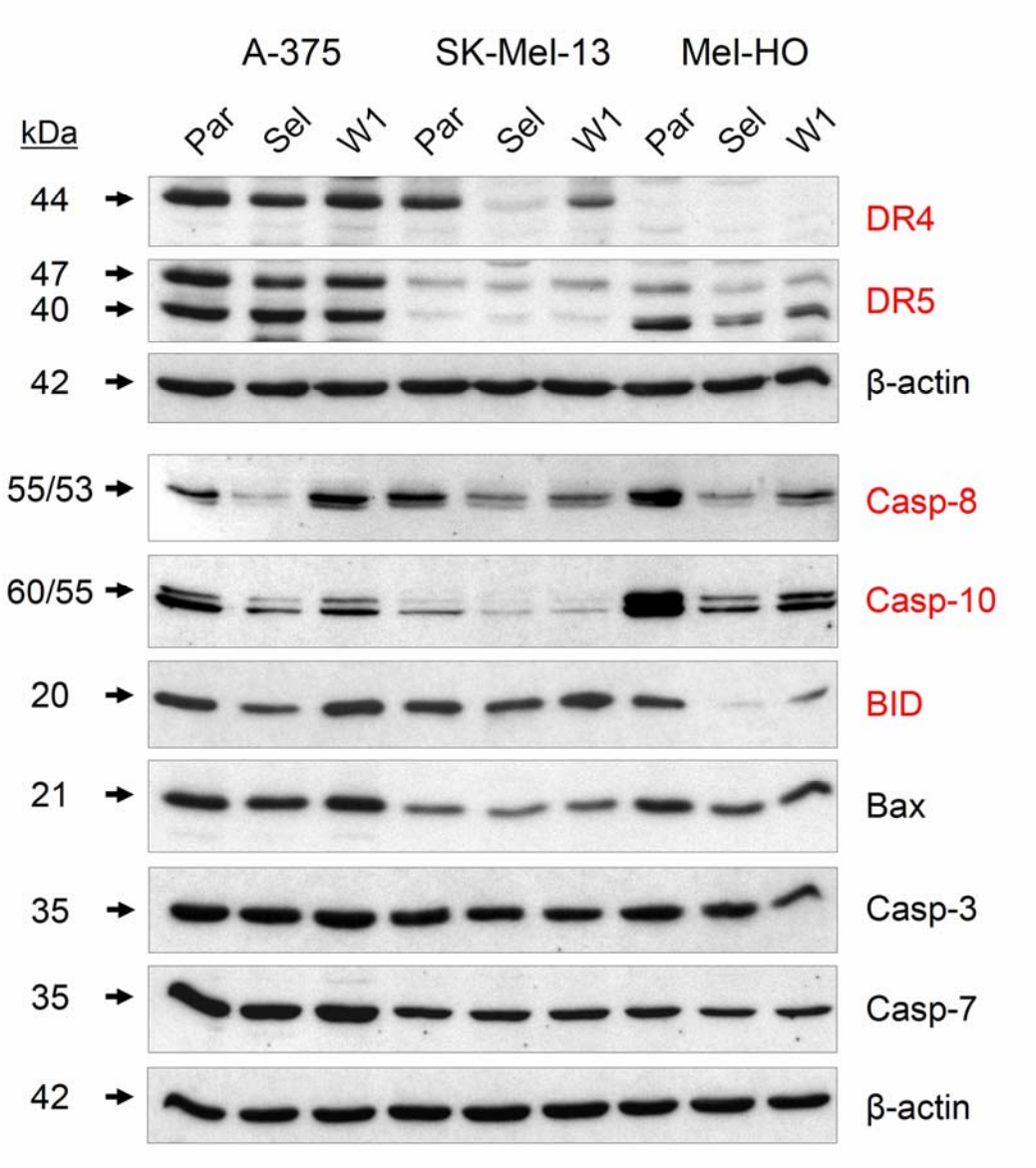

Figure 3.21. Reduced levels of initiator caspases, DR4 and Bid in TRAIL-resistant melanoma cells

The expression of proapoptotic proteins as DR4, DR5, caspase-8, caspase-10, caspase-3, caspase-7, Bid and Bax were investigated by Western blot analysis in parent cells (Par), TRAIL-resistant subcultures (Sel) and cultures collected one month after TRAIL withdrawal (W1). Equal protein amounts (40 µg per lane) were loaded. Two independent experiments for each cell line revealed highly similar results.

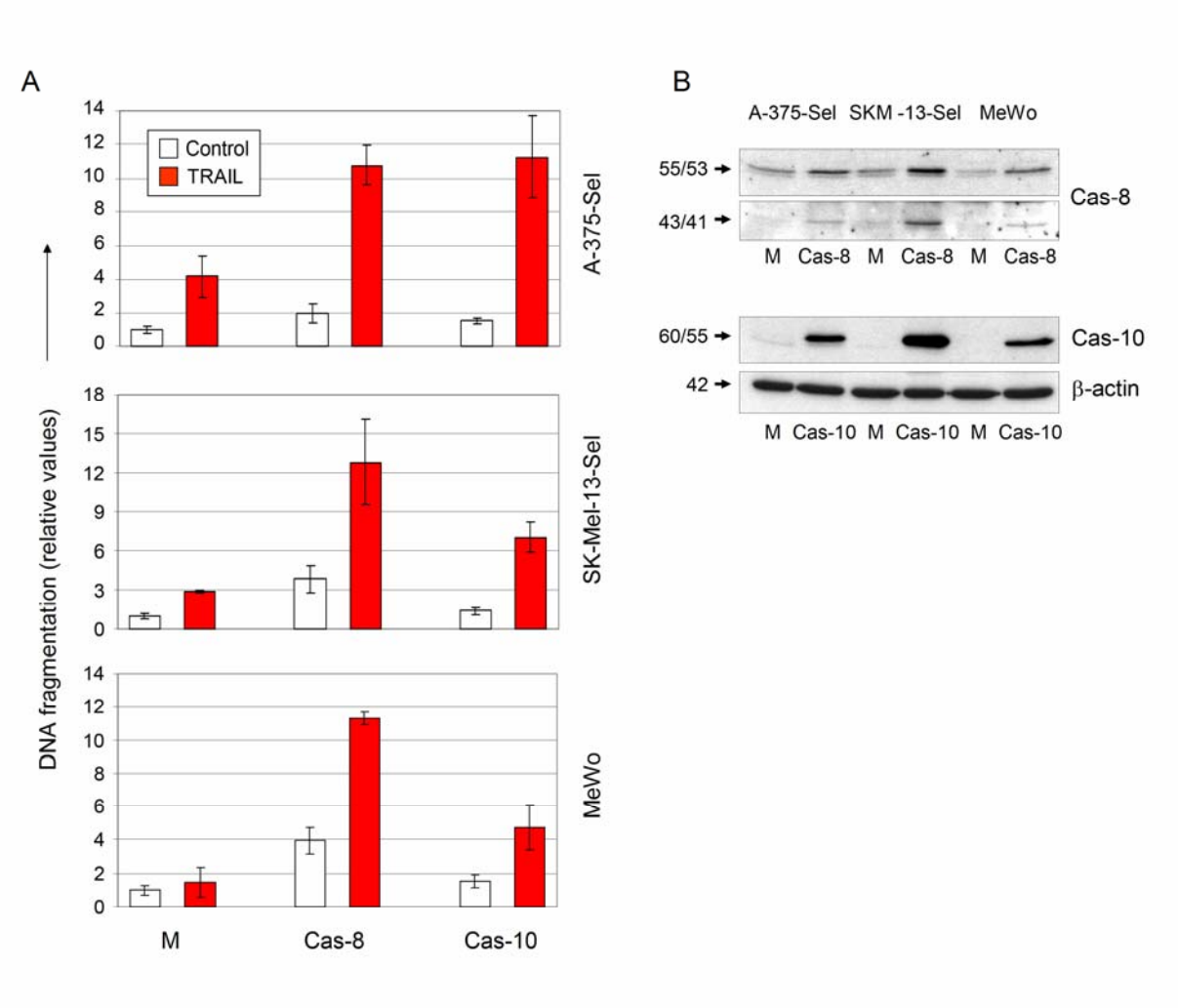

Figure 3.22. Restoration of TRAIL sensitivity by overexpression of caspase-8, and caspase -10

(A) Selected, TRAIL-resistant melanoma cell populations (A-375-Sel and SK-Mel-13-Sel) as well as the TRAIL-resistant parental cell line (MeWo) were investigated. Cells were transiently transfected with expression plasmids encoding for caspase-8 (Csp-8) or caspase-10 (Csp-10), and were compared to mock-transfected controls (M). TRAIL treatment (20 ng/ml) started 48 hours after transfection, and DNA fragmentation rates were determined 6 h later. Untreated mock controls were set to 1 for each cell line. Mean values and SD of three independent experiments each performed in triplicates are shown.
(B) Transfection efficiency was monitored by Western blot analysis. Equal protein amounts (40 µg per lane), prepared 48 hours after transfection, were loaded for determination of overexpression of the respective proteins. Mock-transfected cells were enclosed as controls (M). Two independent series of experiments were performed for each cell line, which revealed highly similar results.

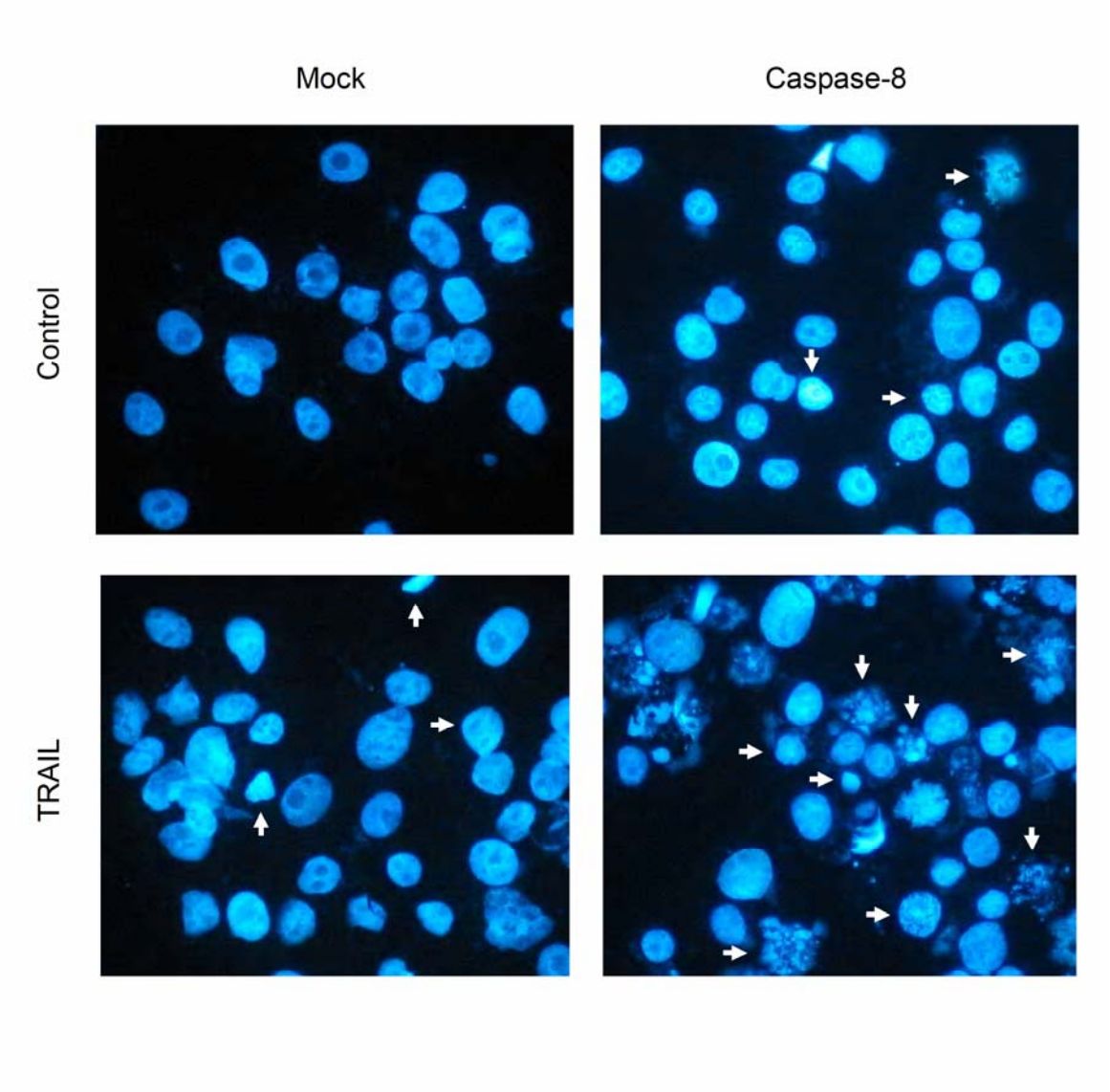

Figure 3.23. After overexpression of caspase-8, TRAIL causes high nuclear condensation and fragmentation in A-375-selected melanoma cells

Nuclear condensation and fragmentation were visualized by Hoechst-33258 staining. A-375-selected cells were transiently transfected with expression plasmids encoding for caspase-8 and with mock-plasmid. TRAIL treatment (20 ng/ml) was started 48 hours after transfection. After another 6 h, cells were transferred to microscope slides by cytospin and were stained with Hoechst dye.

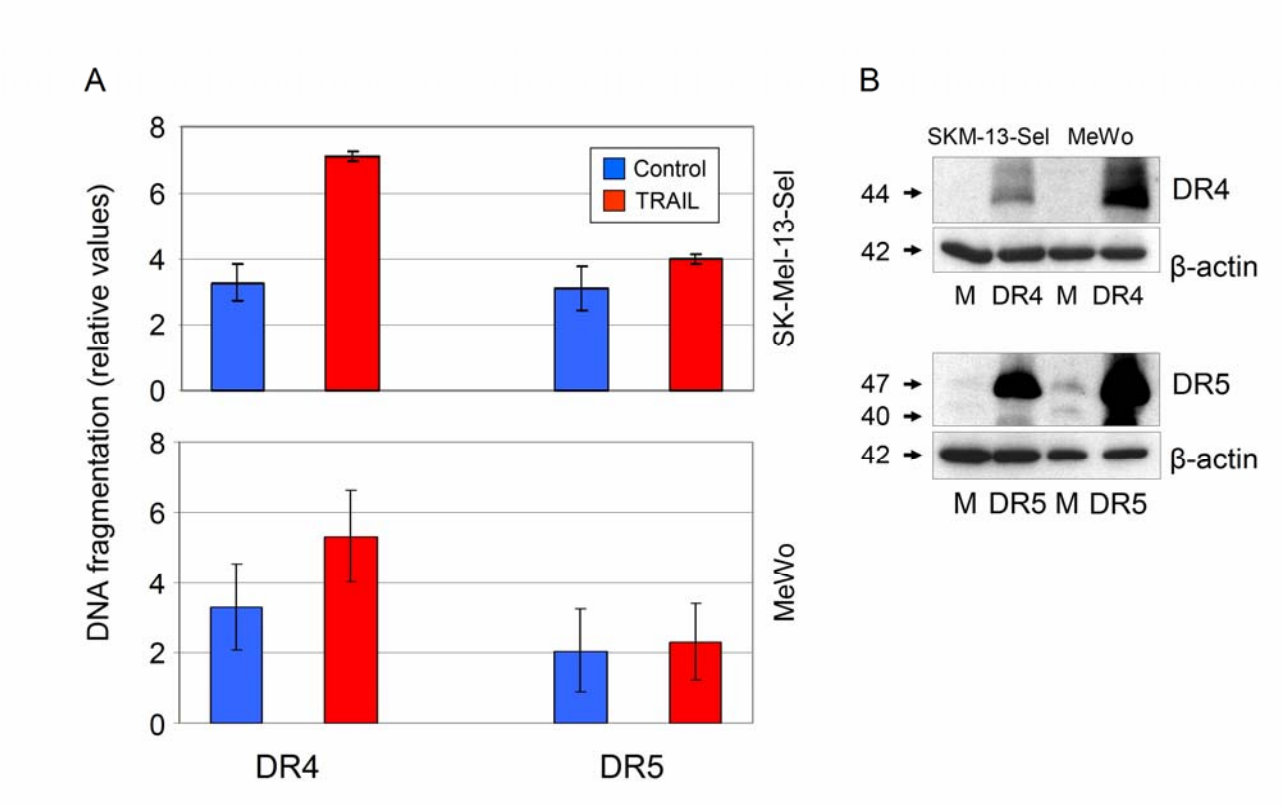

Figure 3.24. Overexpression of DR4 but not of DR5 results in restoration of TRAIL sensitivity in SK-Mel-13-selected and in parent MeWo

(A) Selected, TRAIL-resistant melanoma cell populations (A-375-Sel and SK-Mel-13-Sel) as well as a TRAIL-resistant parental cell line (MeWo) were investigated. Cells were transiently transfected with expression plasmids encoding for DR4 and DR5 respectively. TRAIL treatment (20 ng/ml) started 48 hours after transfection, and DNA fragmentation rates were determined 6 h later. Untreated mock controls were set to 1 for each cell line. Mean values and SD of three independent experiments each performed in triplicates are shown.
(B) Transfection efficiency was monitored by Western blot analysis. Equal protein amounts (40 µg per lane), prepared 48 hours after transfection, were loaded for determination of overexpression of the respective proteins. Mock-transfected cells were enclosed as controls (M). Two independent series of experiments were performed for each cell line, which revealed highly similar results.