Sensitization of Melanoma Cells for Death Ligand TRAIL Is Based on Cell Cycle Arrest, ROS Production, and Activation of Proapoptotic Bcl-2 Proteins

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The death ligand TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) represents a promising strategy for melanoma due to significant expression of TRAIL receptor 1 in melanoma metastases and high TRAIL sensitivity through this receptor. However, prevalent and inducible resistance are limiting its clinical use. In previous work, we and others have described multiple strategies leading to TRAIL sensitization; however, the common principles of these strategies remained elusive. Here, we demonstrate in melanoma cell lines (TRAIL-sensitive, TRAIL-resistant, and TRAIL-selected cells with acquired resistance) that cell cycle arrest clearly correlates with enhanced TRAIL sensitivity. Cell cycle arrest was induced by high cell confluence, serum starvation, or cyclin-dependent kinase (CDK) 4/6 inhibition. Addressing the signaling pathways revealed disruption of mitochondrial membrane potential and production of reactive oxygen species (ROS) in response to antiproliferative conditions alone. Activation of the proapoptotic Bcl-2 protein Bax and inhibition of apoptosis by Bcl-2 overexpression or by the antioxidant *N*-acetyl cysteine underlined the critical involvement of mitochondrial apoptosis pathways and of ROS, respectively. Most pronounced was the upregulation of small proapoptotic Bcl-2 proteins (Puma and Bcl-x_S). These data provide a general understanding on TRAIL sensitization as well as an alternative view on CDK inhibitors and may suggest selective targeting of melanoma cells by cell cycle inhibition and TRAIL.

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INTRODUCTION

Despite recent development of improved therapeutic strategies, long-term prognosis of metastatic melanoma remains fatal. Direct apoptosis induction appears as an alternative, and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is considered as it allows a selective targeting of cancer cells (Walczak *et al.*, 1999; Ashkenazi, 2002; Eberle *et al.*, 2007). TRAIL works via TRAIL receptors 1 and 2 (DR4 and DR5), and we previously demonstrated DR4 expression in melanoma metastases and high sensitivity through this receptor (Kurbanov *et al.*, 2007).

Apoptosis induction is explained by two major signaling pathways. Whereas extrinsic pathways are initiated by death ligands triggering activation of death receptors and initiator caspases-8 and -10 (Krammer *et al.*, 2007), intrinsic mitochondrial apoptosis pathways result from cellular stress as by chemotherapeutics. Characteristic features are depolarization of mitochondrial membrane potential ($\Delta\psi m$), release of mitochondrial factors and activation of initiator caspase-9 (Jin and El-Deiry, 2005). Initiator caspases activate effector caspase-3, which irreversibly triggers cell death (Fischer *et al.*, 2003).

Mitochondrial apoptosis is critically controlled by Bcl-2 proteins, as antiapoptotic Bcl-2, Mcl-1, and Bcl-x_L, proapoptotic multidomain proteins Bax and Bak, proapoptotic BH3-only proteins as Bim and Puma, as well as proapoptotic Bcl-x_S, an alternative splice form of Bcl-x_L (Chipuk *et al.*, 2010; Tait and Green, 2010; Plötz *et al.*, 2012). Bax activation can be monitored by conformational changes detectable by N-terminus-specific antibodies (Westphal *et al.*, 2011), and Bax activation was shown to precede sensitization of melanoma cells for TRAIL (Berger *et al.*, 2013; Quast *et al.*, 2013). Other proapoptotic pathways are related to the production of reactive oxygen species (ROS; Orrenius, 2007), which appeared as particularly effective in melanoma cells (Franke *et al.*, 2010; Quast *et al.*, 2013).

In clinical trials, the effects of TRAIL remained rather limited so far, explained by an inducible resistance (Herbst *et al.*, 2010; Soria *et al.*, 2011). This is also seen in cultured melanoma cells, and TRAIL-selected cell lines have been established (Zhang *et al.*, 2006; Kurbanov *et al.*, 2007).

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Abbreviations: CDK, cyclin-dependent kinase; FCS, fetal calf serum; HD, high density; LD, low density; ROS, reactive oxygen species; siRNA, small interfering RNA; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

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Addressing resistance, different strategies were described that could sensitize for TRAIL. Thus for melanoma, inhibitors of several kinases, potassium channels, histone deacetylase, cyclooxygenase 2, natural and inorganic compounds, or irradiation were effective, as recently reviewed (Quast *et al.*, 2014). Because of the highly different nature of these strategies, the common principles of TRAIL sensitization remained largely elusive.

Growth of normal cells is tightly regulated by cyclins and cyclin-dependent kinases (CDKs; Canavese *et al.*, 2012; Miller and Flaherty, 2014), which are frequently deregulated in cancer (Hanahan and Weinberg, 2011). Cell cycle inhibition therefore represents an important target in cancer therapy. Here, we demonstrate the correlation between cell cycle inhibition and TRAIL sensitivity, suggesting therapeutic strategies by combining CDK inhibitors and TRAIL.

RESULTS

Enhanced sensitivity to TRAIL strongly correlates with cell cycle arrest

For understanding common principles how melanoma cells are sensitized for TRAIL-induced apoptosis, inhibition of cell proliferation was induced by cell confluence, serum starvation, or CDK4/6 inhibition. TRAIL-sensitive cell lines (A-375, SK-Mel-13, and Mel-HO) responded to TRAIL with 4–18% apoptosis at normal seeding (LD, low density: 50–60% confluence, Figure 1a). In contrast, TRAIL was inefficient in TRAIL-selected cells (A-375-TS and SK-Mel-13-TS) and in intrinsically TRAIL-resistant cell lines (MeWo and Mel-2a). Seeding in high density (HD: 100% confluence at treatment) significantly enhanced TRAIL-induced apoptosis in sensitive A-375, SK-Mel-13, and Mel-HO (43, 22, and 11%), as well as in A-375-TS and SK-Mel-13-TS (19 and 12%; Figure 1a). Of note, enhanced sensitivity clearly correlated with G1 cell cycle arrest and reduced cell numbers in G2 (Figure 1b and c).

Only intrinsically resistant cells MeWo and Mel-2a were not sensitized by HD alone. However, further strengthening the antiproliferative conditions by combination of HD and serum deprivation similarly enhanced TRAIL-induced apoptosis in these cells (14–16%). Also in Mel-HO, strongest enhancement of apoptosis (23%) was seen by this combination (Figure 1d). Enhanced sensitivity again correlated with G1 cell cycle arrest (Figure 1e and f).

Proapoptotic activation of caspases, mitochondria, and Bax

For unravelling the pathways mediating enhanced TRAIL sensitivity, caspase activation was investigated by Western blotting. In A-375, some activation (processing) of caspase-8 (41, 43 kDa), caspase-9 (35, 37 kDa), and caspase-3 (15, 17, 20 kDa) was seen by TRAIL at LD conditions, which was lacking in A-375-TS. Caspase processing was strongly enhanced at HD in both cell lines (Figure 2a). The role of caspases was proven by the pancaspase inhibitor Q-VD-OPh, which completely prevented TRAIL-induced apoptosis at LD and HD in A-375 and in A-375-TS (Figure 2b). To distinguish between initial and secondary effects in course of apoptosis, time kinetic analyses were performed. Apoptosis induction by TRAIL started at 6–8 hours at HD and LD, respectively (Figure 2c), which came clearly after the effects shown in the following.

Indicative for proapoptotic mitochondrial pathways, $\Delta\psi m$ was significantly decreased at HD in A-375, A-375-TS, Mel-HO, Mel-2a, and MeWo. It further decreased at HD/– FCS (fetal calf serum), in parallel with enhanced apoptosis (Figure 2d and e). Of note, this happened as a consequence of the antiproliferative conditions alone (without TRAIL-induced apoptosis).

Bax was shown previously to exert decisive roles in regulating TRAIL sensitivity of melanoma cells (Quast *et al.*, 2014). Significant Bax activation, as determined by an N-terminus-specific antibody, was seen in A-375 and in A-375-TS at HD conditions, as well as in Mel-HO, Mel-2a, and MeWo at HD/–FCS conditions (Figure 2f and g).

ROS production

We previously demonstrated the role of ROS in apoptosis control of melanoma cells (Franke *et al.*, 2010; Quast *et al.*, 2013). ROS levels were clearly increased in A-375, A-375-TS, and Mel-HO in response to HD conditions. In contrast, TRAIL largely remained without effect on ROS despite in A-375 at HD, when high ROS levels were further enhanced by TRAIL (1.4-fold). ROS were also enhanced by serum deprivation in Mel-HO, and strongest effects were seen at HD/– FCS (Figure 3a and b). Thus, ROS were produced in response to antiproliferative conditions.

The critical role of ROS was proven by the ROS scavenger *N*-acetyl cysteine. Pretreatment for 2 hours with *N*-acetyl cysteine completely prevented ROS production at HD in A-375 and Mel-HO (Figure 3c and d) and reduced TRAIL-induced apoptosis at HD by 5.1-fold in A-375 and by 2.8-fold in Mel-HO (Figure 3e).

Enhanced TRAIL sensitivity by CDK4/6 inhibition

The critical role of cell cycle inhibition for TRAIL sensitivity was further proven in A-375, A-375-TS, Mel-HO, Mel-2a, and MeWo by the CDK4/6 inhibitor PD-0332991. Alone, it remained without effect on apoptosis at 24 hours but strongly enhanced TRAIL-induced apoptosis in A-375, Mel-HO, and A-375-TS (43, 17 and 19%). Only in intrinsically resistant Mel-2a and MeWo, apoptosis induction by TRAIL/CDK-I remained moderate at +FCS (7% and 8%).

As serum starvation had enhanced cell cycle arrest by HD in parallel with TRAIL sensitivity, it was close to test whether lack of FCS could similarly support CDK inhibition. Indeed, TRAIL sensitivity was significantly enhanced by CDK-I/– FCS in Mel-2a, MeWo, and Mel-HO (15, 16, and 32%; Figure 4a). G1 cell cycle arrest seen in all cells already by CDK inhibition was even more pronounced after additional serum deprivation (Figure 4b). There was a significant correlation between cell cycle arrest and TRAIL sensitivity at different cell culture conditions (correlation coefficient for Mel-HO: 0.87; for MeWo: 0.77).

CDK inhibition resulted in similar effects as seen before for HD conditions. Thus, $\Delta\psi m$ was reduced, and Bax was significantly activated in A-375 also by PD-0332991 at 4 hours (without TRAIL; Figure 4c and d). Only production of

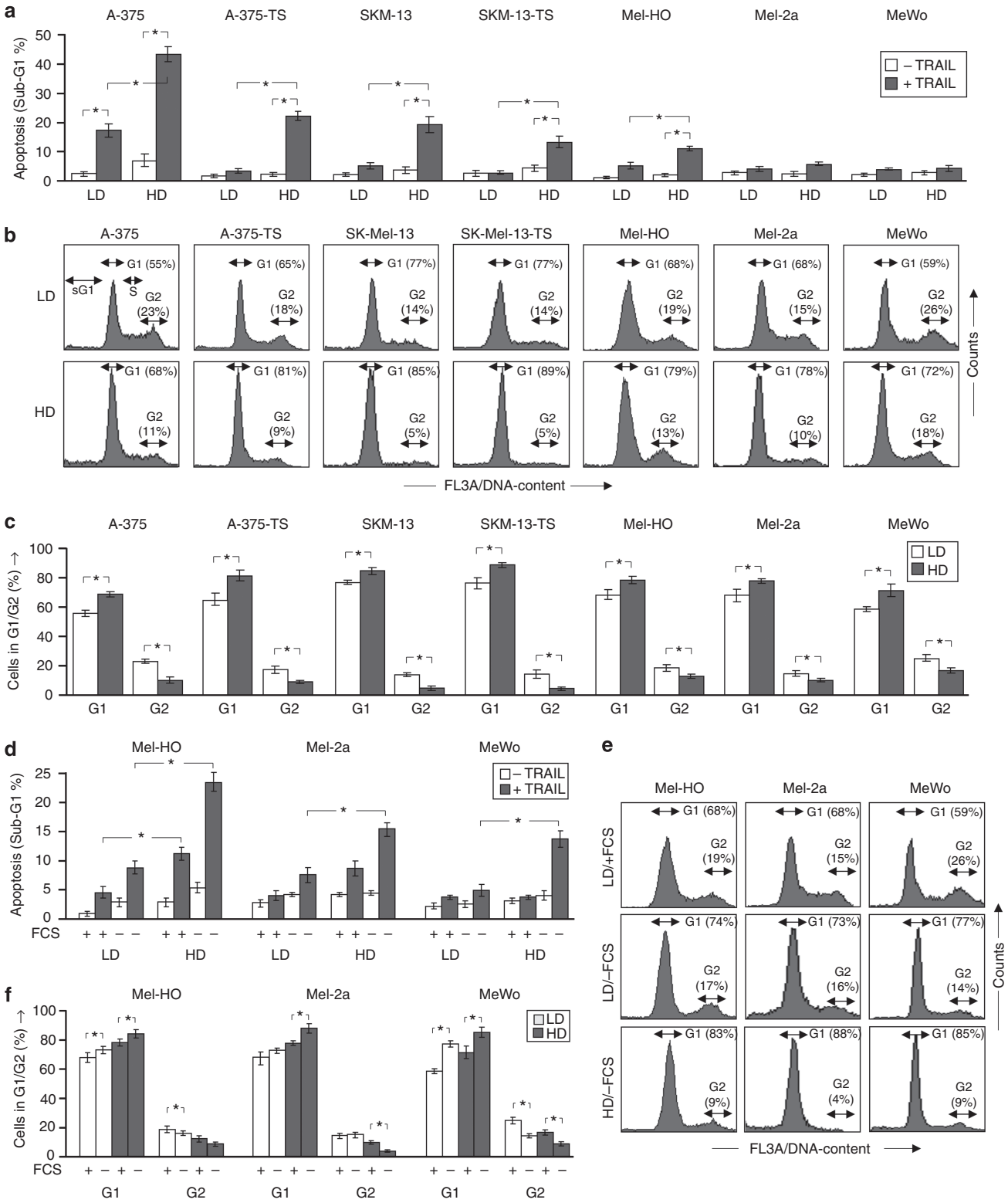

Figure 1. Induced cell cycle arrest correlates with enhanced TRAIL sensitivity. (**a** and **d**) Apoptosis (% of sub-G1 cells) was determined in seven melanoma cell lines in response to TRAIL (20 ng ml^{-1}). Cells were seeded in low (LD) or high density (HD), as well as with or without fetal calf serum (FCS). Means and SDs are of three independent experiments, each consisting of triplicates. Statistical significance (*$P<0.005$) is indicated. (**b** and **e**) Corresponding histograms of cell cycle analyses and % of cells in G1/G2 (mean values). (**c** and **f**) Quantitative cell cycle analyses show % of cells in G1/G2, corresponding to **b** and **e**. Significant changes are indicated by asterisks ($P<0.05$).

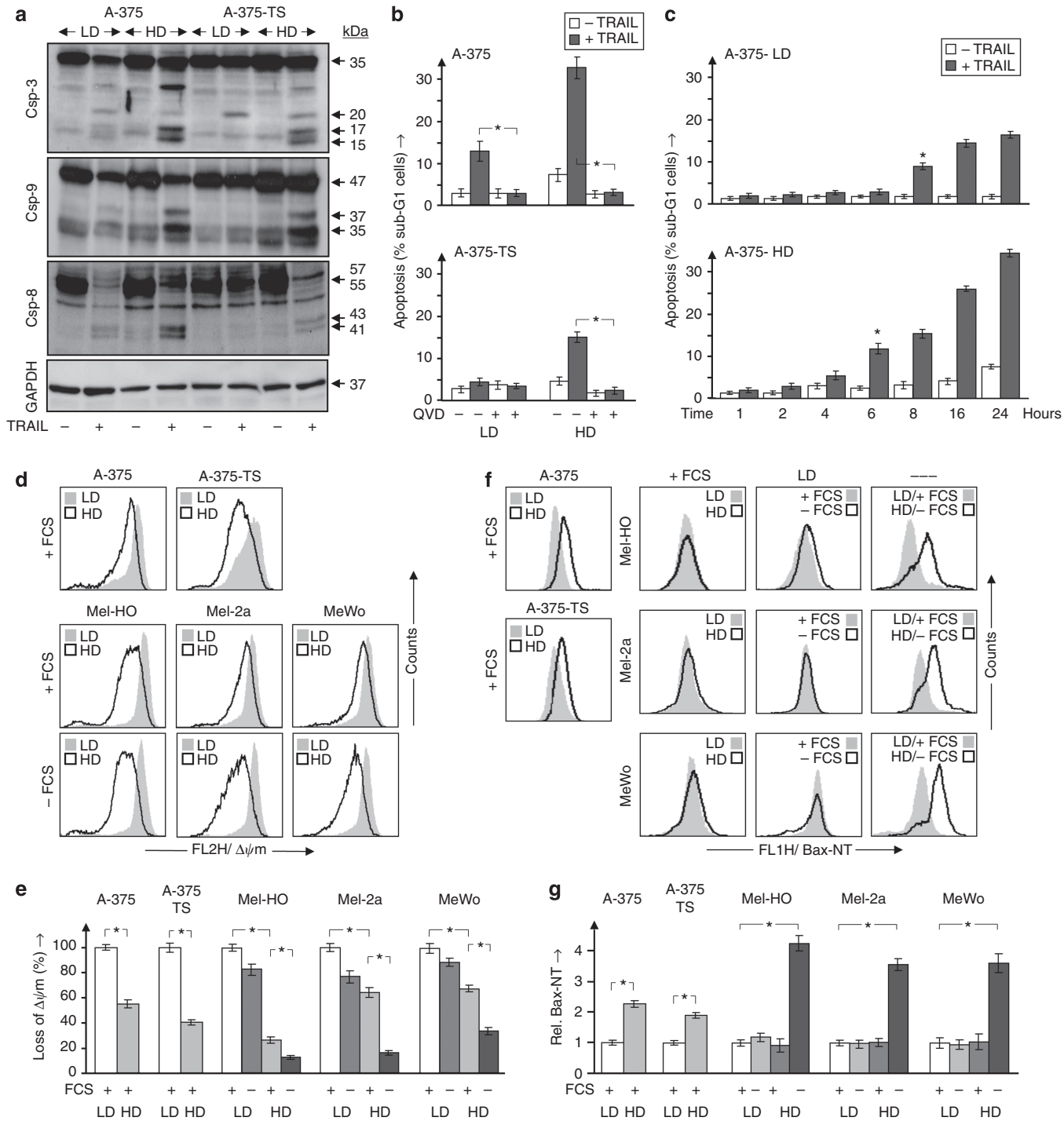

Figure 2. Activation of caspases and mitochondria. (**a**) Processing of caspases-3, -9, and -8 in response to TRAIL (20 ng ml^{-1}) was monitored by Western blotting in cells seeded in low density/high density (LD/HD; loading control: glyceraldehyde-3-phosphate dehydrogenase (GAPDH)). Two independent experiments revealed comparable results. (**b**) Apoptosis (% of sub-G1 cells) in response to TRAIL and the pancaspase inhibitor Q-VD-OPh (QVD, 10 μM, 1 hour pretreatment). (**c**) Time kinetic analyses of apoptosis induced by TRAIL (20 ng ml^{-1}) in A-375 at LD/HD. (**d**) Mitochondrial membrane potential (Δψm) was determined by TMRM^{+} staining. (**f**) Bax activation was determined by Bax-NT antibody in cells at LD/HD ± FCS (fetal calf serum). (**e** and **g**) Quantification of Δψm and activated Bax (each two independent experiments = 6 values, means ± SD). Rel, relative.

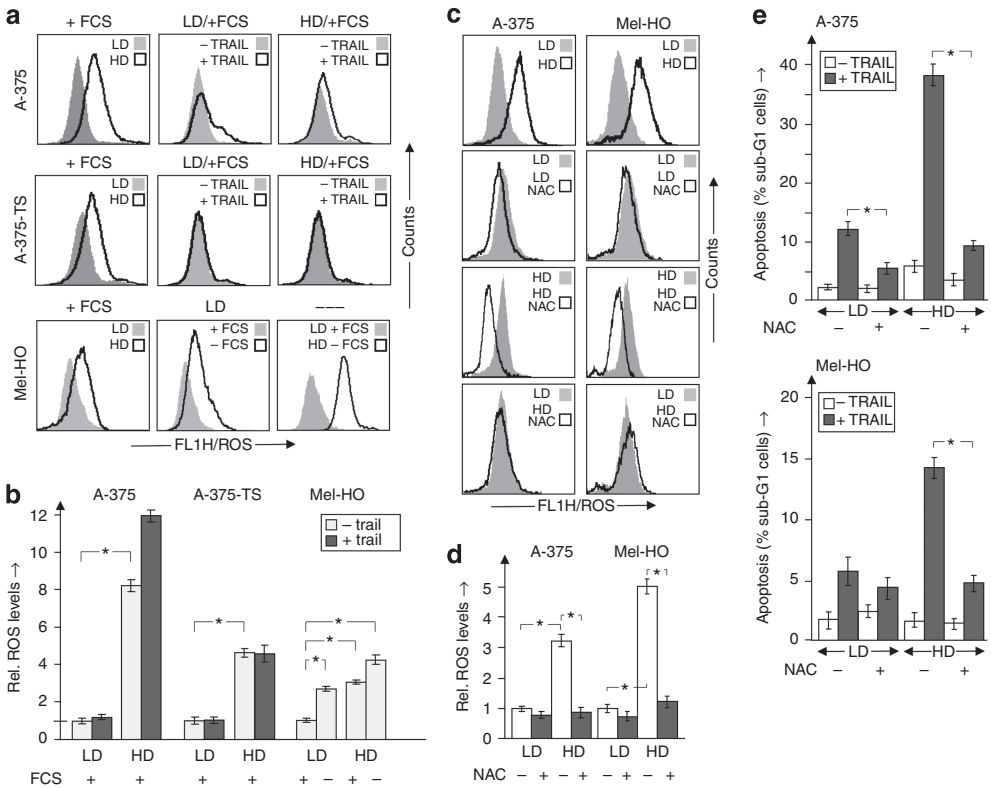

Figure 3. Role of reactive oxygen species (ROS) production. (**a** and **b**) ROS levels were determined by CM-H_2DCFDA staining in cells seeded in low density/high density (LD/HD) ± FCS (fetal calf serum) ± 20 ng ml^{-1} TRAIL. Higher fluorescence corresponds to increased ROS levels. (**c** and **d**) A-375 and Mel-HO cells grown at LD/HD also received the antioxidant *N*-acetylcysteine (NAC, 800 μM, 6 hours). (**e**) Apoptosis (% of sub-G1 cells) was determined in cells grown at LD/HD. Treatment was for 24 hours (TRAIL) and for 26 hours (NAC, 800 μM, 2 hours pretreatment). (**b**, **d** and **e**) Means and SDs of at least two independent experiments (6 values) are shown; statistical significance is indicated (*$P<0.005$). Rel, relative.

ROS in response to PD-0332991 was less pronounced at 4 hours (Figure 4e).

Decisive roles of Bcl-2 and Mcl-1

The dominant role of Bcl-2 became evident by its ectopic overexpression. Thus, TRAIL-induced apoptosis, seen in mock-transfected controls (A-375-pIRES) at LD and HD, was prevented in A-375-Bcl-2, stably transfected for Bcl-2 overexpression (Figure 5a). Also, the G1 arrest induced by HD was diminished by Bcl-2 (Figure 5b). The inhibitory function of Mcl-1 was seen by Mcl-1 knockdown, which significantly increased TRAIL responsiveness in A-375 and A-375-TS both at LD and HD (Figure 5c).

As parental cells, A-375-pIRES responded with significant loss of $\Delta\psi$m to different antiproliferative conditions, which was strongly diminished by Bcl-2 overexpression (Figure 5d and f) underlining the role of mitochondrial activation for TRAIL sensitivity. In clear contrast, ROS production in response to HD and HD/−FCS was not prevented by Bcl-2, rather appeared as even enhanced (Figure 5e and g). This suggested two independent pathways.

Upregulation of small proapoptotic Bcl-2 proteins

Mitochondrial proapoptotic activation was suggestive to search for changes at the expression level of pro- and antiapoptotic Bcl-2 proteins. No changes were obtained by Western blotting for antiapoptotic Bcl-2, Mcl-1, and Bcl-x_L or for proapoptotic multidomain proteins Bax and Bak, when comparing HD and LD conditions. However, when considering small proapoptotic Bcl-2 proteins, which may act as triggers in apoptosis control, significant changes became obvious. Thus, the BH3-only protein Puma was significantly upregulated by HD conditions in A-375, A-375-TS, SK-Mel-13, SK-Mel-13-TS, and Mel-HO. Also Bcl-x_S, the proapoptotic splice variant of Bcl-x_L, was upregulated in SK-Mel-13 and SK-Mel-13-TS by HD conditions, as well as in Mel-HO by HD/−FCS conditions (Figure 6a).

The decisive role of Puma was proven by small interfering RNA (siRNA) strategies. Thus, two distinct siRNA sequences revealed identical results, namely complete knockdown of Puma (Figure 6b), as well as a significant reduction in TRAIL-induced apoptosis at HD in A-375 and A-375-TS (Figure 6c). Downregulation of apoptosis was 1.9- to 4.9-fold, thus clearly underlining the role of Bcl-2 proteins in this response.

DISCUSSION

The death ligand TRAIL represents a promising therapeutic strategy, as apoptosis is induced in a number of tumor cells while normal cells are largely spared (Walczak *et al.*, 1999; Ashkenazi, 2002). Clinical trials have been carried out in non-

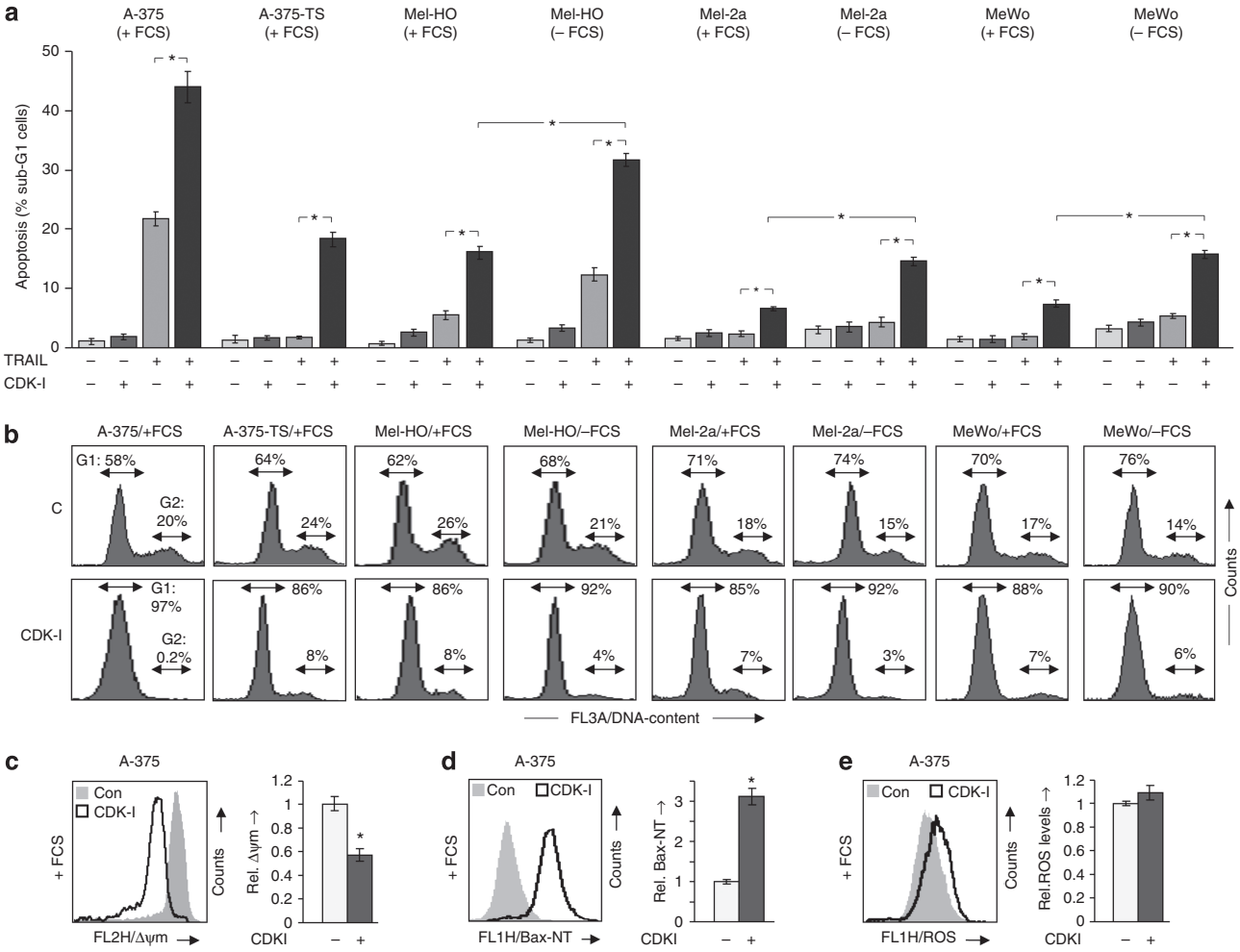

Figure 4. CDK 4/6 inhibitor enhances TRAIL sensitivity. (**a**) Apoptotic cells (sub-G1, in %) were determined by cell cycle analysis after treatment with TRAIL (20 ng ml^{-1}, 24 hours) and PD-0332991 (2 μM, 48 hours, preincubation for 24 hours). Means and SDs of at least two independent experiments (6 values) are shown; statistical significance is indicated (*$P<0.005$). (**b**) Corresponding histograms of cell cycle analyses and % of cells in G1/G2 (mean values). (**c**, **d** and **e**) A-375 cells treated with PD-0332991 (2 μM, 4 hours) are compared with non-treated controls. Right panels, quantification of each two independent experiments (6 values, means ± SD) (**c**) $\Delta\psi m$ was determined by TMRM$^+$ staining. (**d**) Bax activation was determined by Bax-NT antibody. (**e**) Reactive oxygen species (ROS) levels were determined by CM-H_2DCFDA staining. Rel, relative.

Hodgkin's lymphoma, non-small cell lung cancer, and in patients with liver metastases of different origin (Herbst *et al.*, 2010; Younes *et al.*, 2010; Soria *et al.*, 2011). TRAIL should be considered for melanoma because of significant DR4 expression in melanoma metastasis and DR4-mediated TRAIL sensitivity in melanoma cells (Kurbanov *et al.*, 2005). However, inducible and intrinsic TRAIL resistance were reported in different cancer cell models and may explain only limited clinical efficacy (Graves *et al.*, 2014). Thus, 30% of melanoma cell lines reveal intrinsic resistance, whereas TRAIL-sensitive cells may acquire resistance upon treatment (Kurbanov *et al.*, 2007).

Addressing this limitation, different strategies have been reported for enhancing TRAIL sensitivity of tumor cells. In melanoma, these enclosed multiple kinase inhibitors targeting protein kinase C (Gillespie *et al.*, 2005), c-Kit (Hamai *et al.*, 2006), inhibitor of NF-kappaB kinase/nuclear factor kappaB (Berger *et al.*, 2013), phosphatidylinositol 3-kinase/protein kinase B (Quast *et al.*, 2013), and mitogen-activated protein kinases (Berger *et al.*, 2014). Also, inhibitors of potassium channels (Quast *et al.*, 2012; Suzuki *et al.*, 2012), cellular metabolism (Liu *et al.*, 2009; Qin *et al.*, 2010), histone deacetylase (Zhang *et al.*, 2003), and cyclooxygenase 2 (Tse *et al.*, 2014) were effective as well as natural compounds as picropodophyllin (Karasic *et al.*, 2010), indirubin (Berger *et al.*, 2011), and cryptotanshinone (Tse *et al.*, 2013) or inorganic compounds as sodium arsenite (Ivanov and Hei, 2006; Ivanov and Hei, 2011). Because of this extremely high diversity, the pathways of melanoma sensitization for TRAIL remained elusive. The identification of the common end path would allow more selective targeting of cancer cells by TRAIL.

Uncontrolled cell proliferation represents a hallmark of cancer underlined by frequent mutations in cell cycle regulators (Hanahan and Weinberg, 2011). The cell cycle is regulated primarily at G1/S and G2/M phase transition points

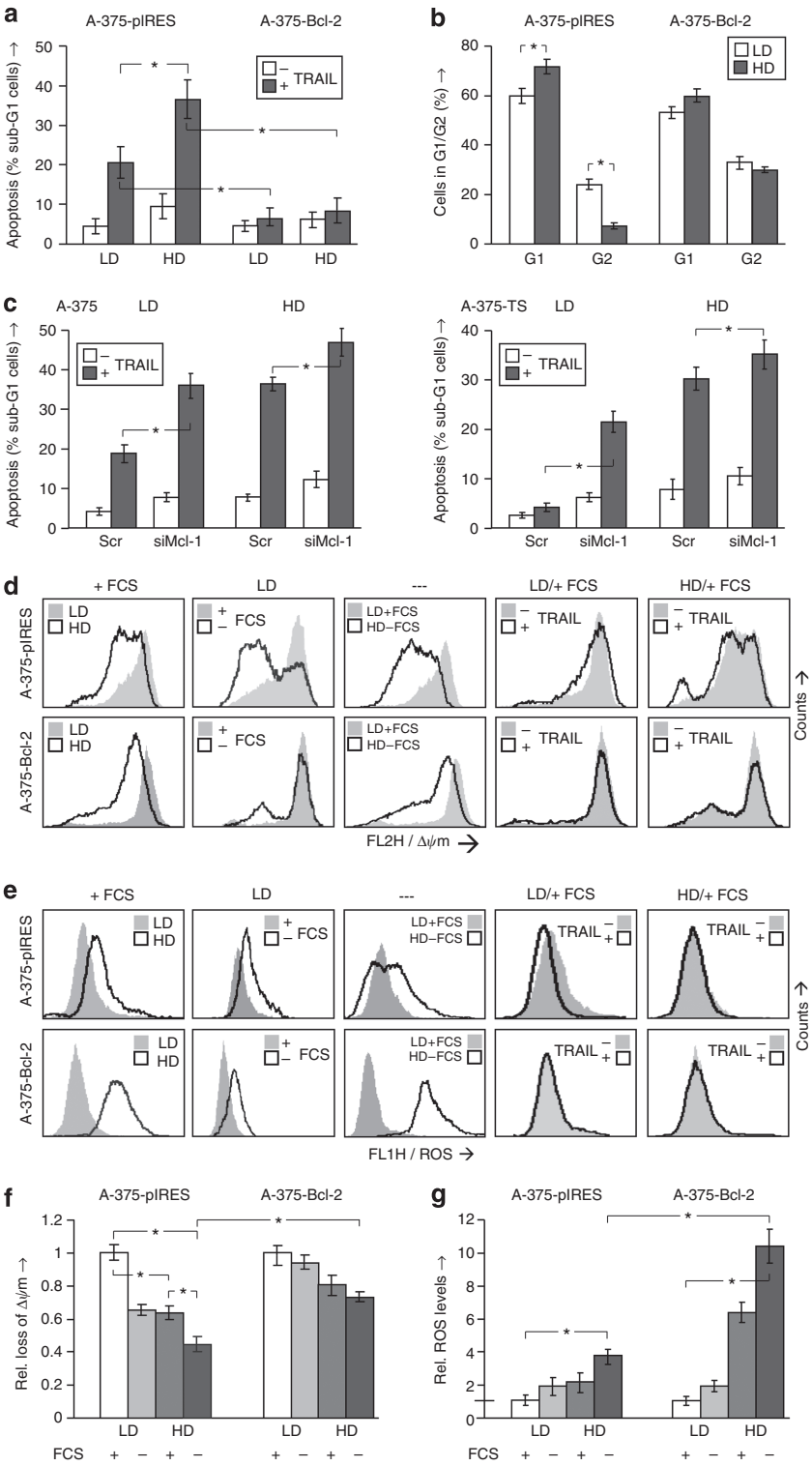

Figure 5. Effects of Bcl-2 and Mcl-1. (**a**) TRAIL-induced apoptosis (sub-G1 cells, %) was investigated in A-375 stably transfected with Bcl-2 (A-375-Bcl-2) and in mock-transfected controls (A-375-pIRES) seeded in low density/high density (LD/HD). Statistical significance is indicated (*$P<0.005$). (**b**) Quantification of cell cycle analyses (% of cells in G1/G2, mean values). (**c**) Impact of Mcl-1 on TRAIL sensitivity (20 ng ml^{-1}, 24 hours) was determined in cells after small interfering RNA-mediated knockdown and compared with respective mock controls. (**d**) Mitochondrial membrane potential ($\Delta\psi m$) was determined by TMRM^{+} staining. (**e**) ROS levels were determined by CM-H_2DCFDA staining. (**f**) $\Delta\psi m$ and (**g**) ROS levels were quantified (each two independent experiments, 6 values; *$P<0.05$). FCS, fetal calf serum; Rel, relative.

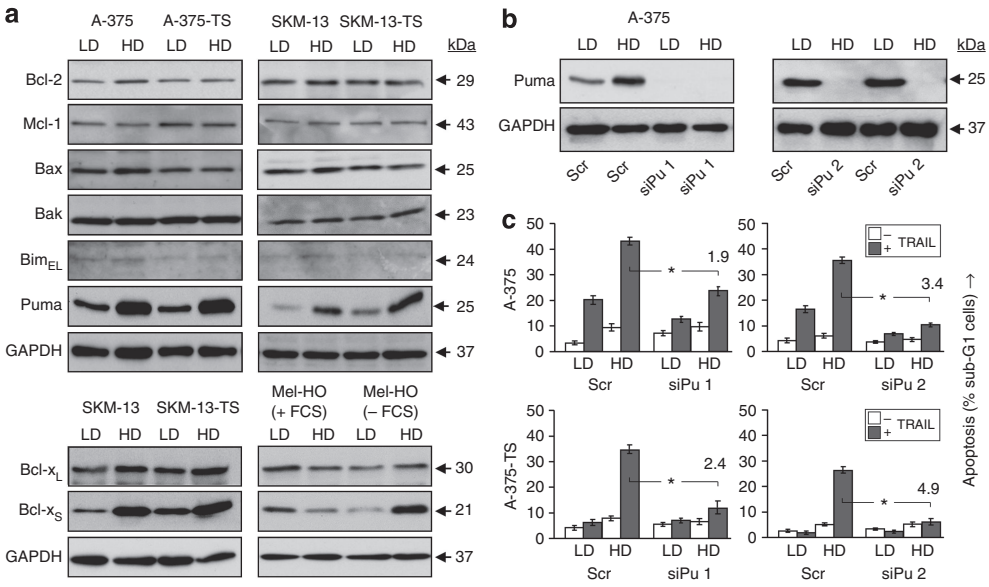

Figure 6. Regulation of Bcl-2 proteins. (**a**) Expression of Bcl-2 proteins in cells grown at low density/high density (LD/HD; Western blotting, loading control: glyceraldehyde-3-phosphate dehydrogenase (GAPDH)). Mel-HO cells were cultivated with/without FCS whereas all other cultures contained FCS. Two independent experiments showed comparable results. (**b**) Downregulation of Puma (25 kDa) by two distinct small interfering RNA (siRNA) sequences (siPu 1/2) as compared with controls transfected with scrambled RNA (Scr; A-375, Western blotting; loading control: GAPDH). (**c**) Apoptosis (sub-G1 cells, %) in response to TRAIL (20 ng ml^{-1}, 24 hours) in cells grown at LD/HD, after siRNA-mediated Puma knockdown (siPu 1/2), as compared with mock controls transfected with scrambled RNA (Scr; mean values ± SDs of two independent experiments, 6 values). Statistical significance of suppressed apoptosis is indicated (*$P < 0.005$).

by cyclins and CDKs (Webster, 1998; Canavese *et al.*, 2012). This regulation is linked to environmental conditions as cell contact inhibition (high cell density) and lack of growth factors (serum deprivation) as applied here.

There is the obvious need for growing cells to coordinate cell proliferation and apoptosis. Thus, antiapoptotic signals frequently support cell proliferation, as reported for some Bcl-2 proteins (Maddika *et al.*, 2007; Danial, 2008; Plötz and Eberle, 2014). The present data suggest enhanced TRAIL sensitivity as a result of cell cycle inhibition. In fact, the degree of cell cycle arrest clearly correlated with enhanced TRAIL sensitivity.

Also, several previously reported strategies for TRAIL sensitization induced cell cycle arrest e.g., inhibition of ion channels or BRAF resulted in G1 arrest (Quast *et al.*, 2012; Berger *et al.*, 2014), whereas an I-κB kinase inhibitor and the natural compound indirubin resulted in G2 arrest (Berger *et al.*, 2011; 2013). Cell cycle arrest therefore appears as the common end path of the different strategies leading to TRAIL sensitization. Enhanced TRAIL sensitivity thus appears as less dependent on a specific pathway but results from cell cycle inhibition in general. This explains why so many different strategies were successful, and it may open improved perspectives for TRAIL-based therapy. Of course, used strategies as particularly serum starvation are highly compex, and different factors in the serum may contribute. Nevertheless, the parallel findings obtained by different strategies strongly support the hypothesis.

Cell cycle inhibition was associated with ROS production. As demonstrated in previous work, ROS represent a distinct proapoptotic pathway in melanoma cells, independent of caspases and independent of the control by Bcl-2 (Franke *et al.*, 2010; Quast *et al.*, 2013). ROS may also inhibit cell proliferation, which was related to upregulation of p21 and Chk1 (Boonstra and Post, 2004; Verbon *et al.*, 2012). ROS production significantly contributed to TRAIL sensitivity, proven by ROS scavenging.

Second, mitochondrial proapoptotic activation seen by loss of mitochondrial membrane potential was characteristic for TRAIL sensitization. These pathways are controlled by pro- and antiapoptotic Bcl-2 proteins. According to present models, Bax and Bak drive the release of proapoptotic mitochondrial factors into the cyctosol. They are neutralized through heterodimerization by antiapoptotic Bcl-2 proteins (e.g., Bcl-2, Bcl-x$_L$, and Mcl-1), which themselves are neutralized by BH3-only proteins (e.g., Bim and Puma; Willis and Adams, 2005). Decisive roles of Bcl-2 and Bax for death ligand sensitivity of melanoma cells were shown by Bcl-2 overexpression, which prevented sensitivity (Raisova *et al.*, 2001; Quast *et al.*, 2014), and by Bax activation through different inhibitors, which enhanced TRAIL sensitivity (Quast *et al.*, 2012; Berger *et al.*, 2013; Quast *et al.*, 2013; Berger *et al.*, 2014). Also in the present setting, Bcl-2 and Bax played decisive roles. Of note, loss of mitochondrial membrane potential, ROS production, and Bax activation appeared as early steps, which were clearly distinguished from secondary effects in course of apoptosis, further underlining the causal relation.

Bim and Puma are central representatives of the subgroup of BH3-only proteins, as they can antagonize all antiapoptotic Bcl-2 proteins (Willis and Adams, 2005; Plötz *et al.*, 2013). Bim is lacking in melanoma, and its re-expression has a role

in apoptosis upon BRAF inhibition (Beck *et al.*, 2013; Berger *et al.*, 2013; Plötz *et al.*, 2013). Also, Puma expression is reduced in metastatic melanoma as compared with primary tumors, which may partly explain chemoresistance and p53 insensitivity (Karst *et al.*, 2005). Puma upregulation was seen here at HD conditions, and its significance for TRAIL sensitivity was proven by siRNA.

Bcl-x_S, the proapoptotic alternative splice variant of Bcl-x_L, may contribute to apoptosis regulation in melanoma cells and triggers apoptosis via Bak (Hossini and Eberle, 2008; Plötz *et al.*, 2012). Upregulation of Bcl-x_S in course of cell cycle arrest suggests that both the Bax- and Bak-dependent proapoptotic pathways were activated.

In conclusion, we aimed to define common principles of melanoma sensitization for the death ligand TRAIL. The data clearly highlighted the importance of cell cycle arrest, which appears as the common end path of multiple strategies. Production of ROS as well as activation of mitochondrial proapoptotic pathways and of Bcl-2 proteins appear as critical downstream steps. The resulting question concerned the mechanistic link between cell cycle arrest and proapoptotic activation. Here, CDKs are highly suitable candidates, and indeed TRAIL sensitivity could be similarly enhanced by CDK 4/6 inhibition.

CDKs already represent promising targets in cancer therapy, and clinical trials are underway (Canavese *et al.*, 2012). As for melanoma, xenograft growth was reduced by CDK inhibition, and early-stage trials have been initiated with selective CDK4/6 inhibitors (Rizzolio *et al.*, 2010; Kwong *et al.*, 2012; Miller and Flaherty, 2014). Tumor stasis and cellular senescence were reported in melanoma cells, whereas there was little evidence for direct induction of apoptosis (Miller and Flaherty, 2014). This limits the potential use as monotherapy, and combinations appear as necessary. The efficacy of combining CDK inhibitors with a direct proapoptotic effector as TRAIL is demonstrated here.

MATERIALS AND METHODS

Cell culture

Human melanoma cell lines enclosed TRAIL-sensitive (A-375, SK-Mel-13, and Mel-HO) and intrinsically TRAIL-resistant cells (Mel-2a and MeWo; Kurbanov *et al.*, 2005). Sublines with induced TRAIL resistance (SK-Mel-13-TS and A-375-TS) derived from selection with TRAIL (Kurbanov *et al.*, 2007). Other A-375 subclones were stably transfected with a pIRES-Bcl-2 plasmid for Bcl-2 overexpression (A-375-Bcl-2) or with pIRES mock plasmid (A-375-Mock; Raisova *et al.*, 2001). Cells were cultured at 37 °C, 5% CO_2 in DMEM (4.5 g l^{-1} glucose; GIBCO, Invitrogen, Karlsruhe, Germany), supplemented with 10% FCS and antibiotics (Biochrom, Berlin, Germany). TRAIL-selected cells were continuously kept with 5 ng ml^{-1} TRAIL until 24 hours before treatment.

For low (normal) cell density (LD), 5×10^4 and 2×10^5 cells were seeded in 24- and 6-well plates, respectively, whereas for high cell density (HD) 2×10^5 and 8×10^5 cells were seeded, respectively. For serum starvation, the growth medium was exchanged by serum-free DMEM at 24 hours after seeding. Treatment with TRAIL (Alexis, Gruenberg, Germany, ALX-201-073-C020, 20 ng ml^{-1}) started 24 hours later. For CDK4/6 inhibition, PD-0332991 (Active Biochemicals, Bonn, Germany) was applied at 2 μM at 24 hours before TRAIL. For inhibition of apoptosis, the pancaspase inhibitor Q-VD-OPh (MP Biomedicals, Santa Ana, CA) was used at 10 μM, 2 hours before TRAIL.

Cell cycle analysis, apoptosis, and cytotoxicity

Cell cycle analyses and quantification of apoptosis were performed according to Riccardi and Nicoletti (2006). Trypsinized cells were stained with propidium iodide (Sigma-Aldrich, Taufkirchen, Germany; 200 mg ml^{-1}, 1 hour), and cell fractions in G1, G2, S-phase, and sub-G1 were quantified by flow cytometry (FACS Calibur, BD Bioscience, Bedford, MA). Sub-G1 cell fractions correspond to cells with fragmented DNA (apoptotic). Cytotoxicity was determined by measuring L-lactate dehydrogenase activity in cell supernatants, applying a cytotoxicity detection assay (Roche Diagnostics, Penzberg, Germany).

Mitochondrial membrane potential and ROS

Mitochondrial membrane potential ($\Delta\psi m$) was determined with the fluorescent dye $TMRM^+$ (Sigma-Aldrich; 1 mM). Cells were harvested by trypsinization, stained for 15 minutes at 37 °C, and analyzed in phosphate-buffered saline by flow cytometry. For intracellular ROS, attached cells were stained with the fluorescent dye CM-H_2DCFDA (Life Technologies, Fisher Scientific, Waltham, MA; 500 μM) at 2 hours before TRAIL treatment started. Trypsinized cells in phosphate-buffered saline were then analyzed by flow cytometry. As positive control, cells were treated with H_2O_2 (1 mM, 1 hour). For ROS scavenging, *N*-acetylcysteine (Sigma-Aldrich; 800 μM) was applied at 2 hours before TRAIL treatment started or at 6 hours before ROS were quantified.

Expression analyses

For Western blotting, total protein extracts were obtained by cell lysis in 150 mM NaCl, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM pepstatin, 0.5% SDS, 0.5% NP-40, and 10 mM Tris-HCl, pH 7.5. Western blotting on nitrocellulose membranes was performed as described previously (Eberle *et al.*, 2003).

Primary antibodies of Cell Signaling (Danvers, MA): Cleaved caspase-3 (9664, rabbit, 1:10,000), caspase-3 (9662, rabbit, 1:1,000), caspase-8 (9746, mouse, 1:1,000), and caspase-9 (9502, rabbit, 1:1,000). Primary antibodies of Santa Cruz Biotech (Dallas, TX): Mcl-1 (sc-12756, mouse, 1:200), Bcl-2 (sc-492, rabbit; 1:200), Bax (sc-20067, mouse, 1:200), Bak (sc-832, 1:200), Bim (sc-11425, rabbit, 1:200), Bcl-x (sc-1041, rabbit, 1:200), and glyceraldehyde-3-phosphate dehydrogenase (sc-32233, mouse, 1:1,000). Puma antibody was from Abcam (Cambridge, UK, ab33906, rabbit, 1:1,000). Secondary antibodies were as follows: peroxidase-labeled goat anti-rabbit and goat anti-mouse (Dako, Hamburg, Germany; 1:5,000).

Bax activation

For analysis of Bax conformational changes related to its activation (Upton *et al.*, 2007), a primary antibody specific for the Bax N-terminal domain (Bax-NT, rabbit, Upstate, Lake Placid, NY, #06-499) was applied in flow cytometry. 10^5 cells were harvested by trypsinization, fixed in 4% paraformaldehyde, and incubated for 1 hour at 4 °C with Bax-NT antibody (1:1,000) in phosphate-buffered saline/1% FCS/0.1% saponin (for cell permeabilization). After incubation with the secondary antibody (goat anti-rabbit IgG

(H+L)-FITC; Jackson Immuno Research, Newmarket, Suffolk, UK), washing, and resuspension in phosphate-buffered saline/1% FCS, cells were measured by flow cytometry.

siRNA transfection

Transient cell transfection with siRNA was performed in six-well plates at 24 hours after seeding; TRAIL treatment started after another 24 hours. Per 24-well, 20 pmol siRNA+4 µl TurboFect (Fermentas, St Leon-Rot, Germany) were used. The siRNAs for Mcl-1 (sc-35877), Puma (sc-37153), and a scrambled control (sc-37007) derived from Santa Cruz Biotech. Another siRNA for Puma (#12845) derived from Cell Signaling.

Statistical analyses

At least two independent experiments were performed, each consisting of triplicates. Mean values and SDs were calculated by enclosing all individual values (at least 6 values). Statistical significance was proven by Student's *t*-test (normal distribution), and *P*-values of <0.05 were considered as statistically significant. Statistical correlation coefficients were calculated according to Pearson.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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