Paclitaxel promotes a caspase 8-mediated apoptosis via death effector domain association with microtubules

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Abstract

Microtubule-perturbing drugs have become front line chemotherapeutics, inducing cell cycle crisis as a major mechanism of action. However, these agents exhibit pleiotropic effects on cells, and can induce apoptosis via other means. Paclitaxel, a microtubule-stabilizing agent, induces a caspase-dependent apoptosis, though the precise mechanism(s) remain unclear. Here, we used genetic approaches to evaluate the role of caspase 8 in paclitaxel-mediated apoptosis. We observed that caspase 8-expressing cells are more sensitive to paclitaxel than caspase 8-deficient cells. Mechanistically, caspase 8 was found associated with microtubules, and this interaction increased following paclitaxel-treatment. The prodomains (DEDs) of caspase 8 were sufficient for interaction with microtubules, but the caspase 8 holoprotein was required for apoptosis. DED-only forms of caspase 8 were found in both primary and tumor cell lines, associating with perinuclear microtubules and the centrosome. Microtubule-association, and paclitaxel-sensitivity, depends upon a critical lysine (K156) within a microtubule-binding motif (KLD) in DED-b of caspase 8. The results reveal an unexpected pathway of apoptosis mediated by caspase 8.

Keywords

caspase; microtubule organizing center; centrosome; cell survival; death effector domain

Introduction

Microtubule-targeted agents are employed as anti-mitotic drugs. These agents comprise several different classes of molecules, and may induce cell death via more than one mechanism (Gascoigne & Taylor, 2008). Caspase activation (Bayless & Davis, 2004; Kasibhatla et al., 2007; Kim et al., 2008; Petit et al., 2008) and "non-caspase protease" (Mitsui et al., 2001) activities have been reported. Paclitaxel is a microtubule-stabilizing drug linked to the activation of numerous caspases, including caspase 3, 8 and 10 (Park et al., 2004; von Haefen et al., 2003; Wieder et al., 2001). In these cases, apoptosis is blocked

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by broad-spectrum caspase inhibitors, and occurs in a 6–24 hour time period. In contrast the induction of apoptosis following cell cycle crisis is observed between 24 and 72 hours.

It remains unclear whether initiator caspases (8 and 10) actually activate prior to caspase 3 to "initiate" cell death, or become activated as bystanders (von Haefen et al., 2003; Wieder et al., 2001). In the current study, we used a genetic approach to examine the interaction between paclitaxel and caspase 8. We examined the efficacy of paclitaxel at inducing apoptosis among cells in which caspase 8 expression was altered, either via shRNA approaches or by reconstitution of genetically deficient cell lines. The approach permitted an evaluation of the impact of caspase 8 in live cells while avoiding pleiotropic (off-target) effects associated with caspase inhibitors (Rozman-Pungercar et al., 2003).

Caspase 8 is composed of a catalytic domain and a prodomain region, the latter containing two "death effector domains" (DEDs). During maturation of the caspase 8 zymogen (also called procaspase 8), the DEDs are cleaved from the catalytic domain, the catalytic domain dimerizes, and the mature caspase 8 catalytic unit is formed. The sequence of the catalytic domain is highly conserved among the different caspases, while DEDs are present only in caspase 8 and 10, and belong to the "death domain" family composed of proteins that influence cell death and proliferation (Tibbetts et al., 2003; Weber & Vincenz, 2001). DEDs can act as protein "adaptor" domains, interacting with other DED modules and likely with other proteins as well (Tang & Tai, 2007). In the case of caspase 8, DEDs have been implicated in cell proliferation (Kennedy et al., 1999; Pellegrini et al., 2005), the induction of apoptosis (Medema et al., 1997) and prevention of apoptosis (Shikama et al., 2002). DED functions appear dependent upon their expression pattern and subcellular localization (Barnhart et al., 2003; Tibbetts et al., 2003; Yao et al., 2007).

In this respect, caspase 8 is found at a number of different cellular locations. Cell behavior influences caspase 8 targeting. For example, caspase 8 can be found within the lamella of migrating cells (Barbero et al., 2008). Here, we assessed whether perturbation of the cytoskeleton by paclitaxel influenced caspase 8 localization or activity. Surprisingly, we found that paclitaxel induced caspase 8-dependent cell death, and that mechanistically, the association of the DEDs of caspase 8 with microtubules promoted cell death.

Results

Cells which express caspase 8 exhibit increased sensitivity to paclitaxel

To characterize the role of caspase 8 in cellular susceptibility to paclitaxel, we evaluated the sensitivity of the caspase 8 deficient neuroblastoma cell line, NB7 (Stupack et al., 2006) and cells expressing caspase 8 (Figure 1A and Supplemental Figures 1 and 2). Within 16 hours, paclitaxel induced greater apoptosis among the caspase 8-expressing cells (Figure 1A). We tested apoptosis in Jurkat cells deficient for caspase 8 or reconstituted for caspase 8 expression treated with a microtubule stabilizing agent, such as paclitaxel or a microtubule de-stabilizing agent, such as nocodazole (Beisner et al., 2003) for 6 and 24 hours (prior to induction of mitotic arrest), and determined that the caspase 8-deficient Jurkat cells were less susceptible to paclitaxel-induced killing (Figure 1B and supplemental Figure 1A). By contrast, nocodazole induced similar (low) levels of apoptosis among caspase 8-positive and

caspase 8-negative Jurkat cells following brief exposure that did not induce mitotic arrest (Figure 1B). Similarly, agents which activate alternative apoptosis pathways, such as staurosporine, induce similar levels of apoptosis in cells expressing or lacking caspase 8 (Supplemental Figure 1B, right panel) (Stupack et al., 2006; Teitz et al., 2002). To extend our examination of this acute response, we next treated the cells with paclitaxel or nocodazole for 6 hours ("pulse") to induce a proapoptotic stimulus, and then washed the cells to remove excess drug. This did not influence the survival of caspase-8 negative, as assessed 20h later (Figure 1C). By contrast, a 6h exposure to paclitaxel was sufficient to intitiate an apoptotic response among caspase 8-expressing cells similar to that observed if paclitaxel was not removed (Figure 1C and Supplemental Figure 1B). Thus, stabilization of microtubules by paclitaxel within 6 hours can trigger an early, caspase 8 dependent proapoptotic event (Figure 1C). To asses the generality of the results, we next suppressed the expression of endogenous caspase 8 in NB5 cells. In agreement with the prior studies, knock-down of endogenous caspase 8 within NB5 neuroblastoma cells was found to decrease their susceptibility to paclitaxel (Figure 1D). Similarly, reconstitution of physiological levels of caspase 8 expression in the NB7 cells (NB7C8) (Stupack et al., 2006) promoted paclitaxel induced apoptosis (Figure 1D). Together, these results implicate caspase 8 as playing a causal role in promoting an "early," paclitaxel-mediated apoptotic response.

Caspase 8 associates with microtubules and centrosomes

Caspase 8 can be recruited to a number of distinct subcellular sites, including the peripheral lamella of migrating cells (Barbero et al., 2008) and endosomes of apoptotic (Schneider-Brachert et al., 2004) and nonapoptotic cells (Torres et al., 2008). To assess the influence of paclitaxel on the subcellular distribution of caspase 8, we used confocal image analysis. Endogenous caspase 8 localizes with different cellular regions; however, of particular interest to these studies was an apparent filamentous distribution which colocalized with tubulin in several cell lines (Figure 2A and supplemental Figure 3). This colocalization was increased by 2-fold by co-incubation with the microtubule-stabilizing agent paclitaxel (Figure 2A *middle panels, Figure 2B*, *p<0.01 and supplemental Figure 3*). However, in the presence of the microtubule de-stabilizing agent, nocodazole, this colocalization appeared to decrease (Figure 2A low panels, Figure 2B and Supplemental Figure 3). Microtubule association was exclusive to caspase 8 since we did not observe colocalization of other caspases with microtubules (Figure 2B and data not shown). As paclitaxel acts to stabilize microtubules, this observation raised the notion that the association of caspase 8 with microtubules might contribute to apoptosis.

To evaluate the mechanism by which caspase 8 associates with microtubules, we next used GFP-tagged constructs of the caspase 8 holoprotein, as well as the N and C terminal domains of caspase 8, and assessed their subcellular distribution (Figure 2C). As shown, the N-terminal domains (DEDs) of caspase 8 displayed a filamentous distribution; these filaments colocalized with anti-tubulin antibody (Figure 2D, and Supplemental Figure 4). We were unable to demonstrate significant colocalization with other subcellular compartments, including actin filaments or Golgi. His-tagged DEDs exhibited a similar localization to the GFP-tagged constructs, suggesting that the DEDs, rather than GFP,

mediated the interaction with microtubules (Figure 2C, left panel and Supplemental Figure 4B). In support of these findings, DEDs-GFP, but not GFP alone, immunoprecipitated with tubulin (Supplemental Figure 4C). Notably, the localization of the DEDs in these cells was usually perinuclear, a site where microtubule turnover is less dynamic than in the cell periphery. Together with the observation that paclitaxel increased caspase 8 association with microtubules, the results suggest that DEDs might associate preferentially with "stable" microtubules.

We were aware that DEDs have previously been described to form filaments in cells (Siegel et al., 1998), and that DED-filaments were suggested to form spontaneously via direct DED-DED association. In agreement with this notion, DEDs can aggregate in cell-free systems, (and indeed, we find that DEDs will aggregate following expression in bacterial systems) (DGS, unpublished observations). However, free DEDs have not been described to form filaments. We therefore speculated that interactions promoted by a microtubule scaffold might account for the formation of the "filaments." To directly assess whether microtubules influenced DED filaments, we treated caspase 8-deficient cells expressing the GFP-DEDs with either paclitaxel (to stabilize microfilaments) or with nocodazole (to disrupt microfilaments). Paclitaxel increased the incidence and length of DED microfilaments, as assessed in either live or fixed cells (Figure 3A). By contrast, treatment with nocodazole disrupted DED filaments (Figure 3A), frequently resulting in a punctate distribution of DED (a non-filamentous aggregate). Together, the results were consistent with the notion that (stable) microtubules are important for DED colocalization and "filament" formation.

The DEDs were frequently observed in a characteristic stellate appearance reminiscent of centrosomes (Figure 3B) and we subsequently confirmed that the DEDs were centrosome-associated via cell fractionation and centrosome purification (Figure 3C). Localization of caspase 8 DEDs at the centrosome was not an artifact of expressing GFP-DEDs, since antibody to the caspase 8 DEDs also detected endogenous caspase 8 at the centrosomes (Figure 3C, left panels). Endogenous DEDs also colocalized with centrosomes (Figure 4B and supplemental Figure 5) and were detected within the purified centrosome fraction (Figure 4B, right panel). However, the DEDs are not detected at the centrosomes of cells that do not express caspase 8 (Supplemental Figure 5).

The DEDs of caspase 8 are present in nonapopototic cells

It remained unclear whether the caspase 8 holoprotein interacted with microtubules, or whether the interaction was specific to the maturation-processed DED fragments. The DEDs appeared sufficient, and significantly more efficient, at integrating into filaments (Figure 2C). An immunofluorescence approach using antibodies specific for either the catalytic domain or for the DEDs revealed that DEDs frequently colocalized with the catalytic domain. However, interestingly, we also observed areas where principally DED-staining was observed (Figure 4A). To test this more directly, we next evaluated the localization of DEDs or the caspase 8 catalytic domain with the centrosome (Figure 4B, middle panel). Confocal analysis of the centrosomes revealed staining with a DED-specific antibody in almost all cells, but only rarely with an antibody specific to the catalytic domain. Together, the data suggest that mature DEDs are either more efficient at microtubule association than

the caspase 8 holoprotein or, alternatively, that caspase 8 maturation may follow microtubule association.

These results further suggest that the DEDs might also be produced from endogenously expressed caspase 8. Immunoblot analysis of nonapoptotic COS-7 cells revealed the presence of an amino-terminal DED-containing fragment (Figure 4C). We considered that the DEDs might accumulate as part of a background level of apoptosis within these cell populations; therefore, we looked for a similar accumulation of the C-terminal domain. However, we were consistently unable to detect signs of caspase 8 maturation other than the DEDs (Figure 4C, and unpublished observations). Moreover, the expression of the DED fragment was not unique to COS-7 cells, but was found in monocytes, endothelial and epithelial cells as well as in caspase 8-positive neuroblastoma (Figure 4D). By contrast, DEDs were not detected in caspase 8-deficient cells (Figure 4E). Although there was no significant apoptosis in these populations, we speculated that the DEDs could accumulate as a result of low levels of caspase 8 maturation occurring independent of apoptosis (Gdynia et al., 2007; Su et al., 2005). Since caspase 8 clearance is mediated via ubiquitinylation of the catalytic domain (McDonald & El-Deiry, 2004), the selective retention of mature DEDs appeared reasonable. To explore this possibility, we examined neuroblastoma cells reconstituted with either a catalytically inactive caspase 8 mutant (C360A) or wildtype caspase 8. Although the basal rate of apoptosis in these populations was similar (~3–5%, assessed by FACS), we detected the mature DEDs of caspase 8 selectively in lysates derived from cells reconstituted with active enzyme, and not in those derived from cells expressing the catalytically dead mutant (Figure 4E). Catalytic activity therefore permits an endogenous degree of DED production. However, we cannot discount the possibility that DED-only proteins could accumulate directly, via the expression of caspase 8 isoforms that lack the catalytic domain, such as caspase 8 D, E and G (Scaffidi et al., 1997).

DEDs promote caspase 8-dependent apoptosis

We assessed whether association of the DEDs was sufficient to induce apoptosis. We found that simple DED expression in COS-7 or 293 cells was sufficient to induce apoptosis (Figure 5), however, no apoptosis was observed in the caspase 8 deficient NB7 cells. When DEDs were expressed in caspase 8 reconstituted NB7C8 cells, we observed subsequent activation of the caspase 8 holoenzyme and apoptosis (Figure 5). Thus, paclitaxel and DEDs required a pool of catalytically active caspase 8 to promote apoptosis. These results raised the notion that microtubules might serve as a scaffold to recruit and activate caspase 8.

DED-b is critical for microtubule association

Using a truncation mutation approach, we found that DED-b expression was sufficient to promote microtubule binding (Figure 6A, table). DED-a did not localize to microtubules. We next extended this result using a point mutagenesis strategy, specifically targeting charged residues within the inter-helix loops of DED-b (Figure 6A). As shown, the K156R mutation in the helix 4-helix 5 loop of DED-b disrupted recruitment to microtubules (Figure 6A and B). In contrast, mutation of residues in other loops had no effect on microtubule association of the DEDs. Interestingly, the K156R mutation falls within a microtubule binding motif (KLD) in the loop/turn between helices 4 and 5 in DED-b. This motif is

shared with the "IR1-2" region of the microtubule-binding proteins Tau (Goode & Feinstein, 1994) and MAP2C/D, as well as the microtubule "cleft binding finger" of M type kinesins (Blaineau et al., 2007). Mutation of the lysine in this motif also inhibits microtubule association mediated by the IR1/2 region of Tau (Goode & Feinstein, 1994), and tubulin cleft binding by KIF2C (Ogawa et al., 2004).

Confocal microscopy analysis and co-staining for γ tubulin revealed that the K156R mutation blocked association with centrosomes (Figure 6C). Since microtubule association mapped to a distal loop in DED-b, the "DED-binding" RxDL motif would be located at the opposite end of the DEDs in DED-a (Garvey et al., 2002b). This prompted us to consider whether the two DED domains might operate cooperatively to induce apoptosis.

Microtubule accumulation of DEDs promotes the caspase 8 component of paclitaxel-induced apoptosis

Since paclitaxel promotes localization of caspase 8 with microtubules and induces a caspase 8 dependent apoptosis, we hypothesized that DED-association with microtubules might be important to activate a caspase-dependent component of paclitaxel-induced apoptosis. We tested whether differences existed in apoptosis induced by paclitaxel in cells expressing procaspase 8 or those expressing a procaspase 8 mutant deficient in microtubule binding (C8-K156R). Expression of a microtubule-binding mutant of caspase 8 rendered cells resistant to paclitaxel treatment, while cells expressing wild type caspase 8 remained sensitive to paclitaxel-induced, caspase-dependent killing (Figure 7A). These results suggested that microtubule association of caspase 8 might be important for paclitaxel mediated triggering of caspase 8.

We next tested whether DED decoration of microtubules was sufficient to induce apoptosis independent of paclitaxel. In cells lacking caspase 8, neither wt DED nor K156R mutants induced apoptosis. Among, caspase 8-expressing cells, however, the DEDs strongly induced apoptosis and caspase 8 activation, while expression of the KLD mutant DEDs did not (Figure 7B). Together, the results suggested that microtubule association of DEDs was important for subsequent caspase 8 activation.

Testing this via immunofluorescence analysis, we observed a dramatic change in procaspase 8 distribution after expression of DEDs (Figure 7C). Wildtype Caspase 8 was found to be recruited to microtubule-associated DEDs (Figure 7C, left middle panels). Moreover, the recruitment of mutant full length caspase 8 bearing the K156R mutation to microtubules was rescued by coexpression of wildtype DEDs (Figure 7C, right middle panels). The results show that although the K156R mutation prevents direct interaction with microtubules, it does not prevent homotypic interactions (direct DED-DED interactions) known to occur via the DEDa domain (Figure 7C). Consistent with these results, both wt caspase 8 and caspase 8 bearing the K156R mutant induced apoptosis in the presence of wt DEDs (Figure 7D). As expected, neither K156R (not shown) nor wt caspase 8 (Figure 7C, right panels) was found associated with microtubules in the presence of the mutated K156R DED domains.

Based on this, we evaluated whether expression of DEDs would enhance paclitaxel-induced apoptosis. Paclitaxel-mediated apoptosis was promoted by wt DEDs, but not the K156R

mutant (Figure 7E and Supplemental Figure 6). The expression of DEDs displayed no synergy with nocodazole, a microtubule destabilizing drug (interestingly, there was a trend towards decreased apoptosis). These observations indicate that the microtubule-binding function of the DEDs is important for the caspase 8-mediated cell death following acute paclitaxel exposure. Together, our studies suggest that the paclitaxel efficacy may require only an exposure of a few hours to kill some tumor targets (much briefer than that required for mitotic arrest), and further define a new mechanism in which microtubule-scaffolded DEDs contribute to caspase 8 activation.

Discussion

Paclitaxel induces cell cycle arrest and/or crisis, promoting cell death. These studies focused on the early, caspase 8-dependent apoptotic response resulting from the recruitment of mature DEDs to microtubules. Finding that caspase 8 expression results in increased apoptosis, we determined that the caspase dependent response was also microtubule dependent. The accumulation of DEDs on microtubules potentiates caspase 8 recruitment, and promotes caspase activation and cell death. It is conceivable that similar events could occur during the early stages of death receptor mediated killing, wherein an initial modest accumulation of DEDs on microtubules could act to amplify receptor-mediated caspase 8 activation. Indeed, in our hands, expression of DEDs or paclitaxel act synergistically to promote apoptosis via Fas (A.M., D.S. Unpublished Observations). Microtubule stabilizing agents which could promote the microtubule-DED complex would therefore be predicted to amplify ongoing caspase 8 activation, acting as a second surrogate "death inducing signaling complex" (DISC). Such a positive feedback loop would provide one explanation for the synergies observed between taxanes and death receptor ligands (Nimmanapalli et al., 2001; Odoux & Albers, 2004; Odoux et al., 2002; Singh et al., 2003; Zhu et al., 2004). Such a model may also explain why microtubule-destabilizing drugs hinder DR-mediated killing (Feng & Kaplowitz, 2000). The capacity for taxanes to mediate proapopotic action among caspase 8 expressing tumors without requiring cell cycle arrest could influence efficacy among tumors, particularly those with compromised vascular perfusion.

The precise molecular mechanism of DED association with the microtubules remains unclear. The interaction appears dependent upon a KLD motif which is also found in class II microtubule binding proteins, M-type kinesins and some DEDs (Al-Bassam et al., 2003; Al-Bassam et al., 2002; Blaineau et al., 2007; Goode & Feinstein, 1994; Ogawa et al., 2004). The motif occurs at the opposite end of the caspase 8 DEDs from the RxDL motif responsible for DED-DED interaction (Carrington et al., 2006). The KLD motif is absent from viral DEDs that protect against caspase-8 mediated killing, such as MC159 (Garvey et al., 2002a). Importantly, K156R procaspase 8 mutants which could not bind microtubules directly could still be rescued in their ability to induce apoptosis by wild type DEDs.

The results also suggest that previously described DED "death effector filaments" are likely microtubule-associated DEDs (Siegel et al., 1998). Recent studies identifying the regions of DEDs that associate (Carrington et al., 2006; Garvey et al., 2002b) indeed imply that "excess" DEDs might be expected to compete with procaspase 8 for binding sites (Shikama et al., 2002), and as a steric consequence, could actually preclude procaspase clustering and

activation (Boatright et al., 2003). By contrast, a microtubule scaffold should promote apoptosis more efficiently, since it could adsorb the DED-b domain directly while allowing recruitment of additional caspase 8 via the DED-a RxDL motif.

As pointed out recently by Gascoigne and Taylor (Gascoigne & Taylor, 2008) microtubule targeting drugs have been used broadly in chemotherapeutic applications, despite the fact that we know little about their mechanism of cell killing. In their studies, they demonstrated that prolonged exposure to antimitotic drugs could potentially result in multiple different cell fates. Many cells exhibited heterogenous responses, possibly due to differences in drug target binding, multidrug resistance/trasnporters, or possibly the expression of caspases, such as caspase 8. In this regard, others have shown that caspase 8 activation via TRAIL can accelerate apoptosis induced by mitotic checkpoint drugs (Kim et al., 2008). In contrast to our results, however, these studies showed a cooperativity between nococazole and caspase 8 mediated killing, which we do not observe (Figure 1, 7), likely via cleavage of spindle checkpoint proteins. Such differences may be explained, in part, by the fact that our studies focused on the acute cell reaction to pactlitaxel and nocodazole. However, we did observe cooperativity between a Fas agonist and paclitaxel (Supplemental Figure 7), so activation of caspase 8 may be a common "enhancing" factor for cell death following microtubule perturbation. Indeed, in this case, microtubule-associated DEDs could act to amplify external apoptosis signals (Supplemental Figure 8).

From the clinical perspective, microtubule-based amplification of caspase 8 activation may contribute to the efficacy of caspase 8-dependent drugs, including the DR agonist TRAIL (Odoux & Albers, 2004), proteosome inhibitors (Calvaruso et al., 2006; Chauhan et al., 2008; Kabore et al., 2006; Miller et al., 2007; Voortman et al., 2007), and, as shown here, taxanes (Nimmanapalli et al., 2001; von Haefen et al., 2003; Wieder et al., 2001). Such a role for caspase 8 in paclitaxel mediated effect may well explain the poor impact of paclitaxel in malignant neuroblastoma, which are generally caspase 8-deficient (Kretschmar et al., 2004; Teitz et al., 2001). However, an improved understanding of the underlying mechanisms by which caspase 8 effects cell death may provide new opportunities to increase efficacy among caspase-8 directed therapies already in clinical use.

Materials and methods

Vectors and constructs

The GFP-caspase 8 constructs have been previously reported (Barbero et al., 2008). K156R, S113A, E127A, and D141A mutations were introduced in DED-GFP using the QuikChange Mutagenesis kit (Stratagene) with appropriate mutagenesis primers. DED myc-His fusion protein was shuttled from GFP-DED into pcDNA3.1 myc-His (Invitrogen).

Cell culture and transfections

Previously described human neuroblastoma cells, deficient or expressing caspase 8 (Stupack et al., 2006), were cultured in RPMI supplemented with 10% fetal bovine serum, glutamine and non-essential amino acids. Human keratinocytes (HaCat), human carcinoma cells (A549) and monkey fibroblasts (COS-7), were cultured in DMEM, supplemented as

previously described (Stupack et al., 2001). Human endothelial cells (HUVECs) were cultured in M199 with endothelial cell supplement, 10% fetal bovine serum, glutamine and minimal essential amino acids. Neuroblastoma cells deficient in caspase 8 were transfected using the Fugene reagent following manufacturer's protocol. Stable cell lines were selected with 500μg/ml G418 (Gibco) and FACS-sorted for GFP-positive cells. Caspase expression was confirmed by immunoblotting with caspase 8 N-terminus (BD Pharmingen) and C-terminus specific antibodies (the clones C5 and C15 monoclonal antibodies, were a gift from M. Peter, University of Chicago, USA). NB7 cells stably transfected with DED-GFP were treated with 600nM nocodazole or 200nM paclitaxel (Sigma) or DMSO (control) overnight. Living and fixed cells were visualized with a 20 or 63x objective using a Nikon Eclipse C1 confocal microscope.

Immunofluorescence studies

Cells attached to coverslips were fixed with 4% PFA and permeabilized in PBS containing 0.1% triton for three minutes and blocked for 60 minutes, at room temperature with 2% BSA in PBS. Cell were stained with antibodies to the amino terminal death effector domain of caspase 8 (BD Pharmingen and Calbiochem), antibodies specific for α tubulin (Abcam & Calbiochem), γ tubulin (Abcam), pericentrin (Abcam), ER (anti-PDI, Stressgen), Golgi (anti-GM130, Transduction labs), vimentin or actin (Sigma), lamin B (Santa Cruz) or mitotracker (Invitrogen). All primary antibodies were used at 1:100 dilution, for two hours at room temperature. After washing several times with PBS, cells were stained for two hours at room temperature, with secondary antibody specific for mouse, rabbit or goat (Invitrogen), as appropriate, diluted 1:300. In some cases, cells were coincubated with the blue DNA dye TOPRO-3 (1:1000) (Invitrogen). Samples were mounted in Vectashield hard-set mounting media (Vector Laboratories) and imaged on a Nikon Eclipse C1 confocal microscope with a 1.4 NA 60x oil-immersion lens, using minimum pinhole (30μm). For immunoblot analysis and immunofluorescence, antibodies specific for the amino terminal death effector domain (BD Pharmingen and Calbiochem) or the carboxy terminal catalytic domain (clones C5 and C15) (Scaffidi et al. JBC, 1997) as well as a polyclonal antibody we raised to the recombinant catalytic domain of caspase 8 here at UCSD. For demonstration of centrosome recruitment, pixels thresholding (top 20%) was performed. Immunofluorescence were also performed with a mouse monoclonal FADD specific antibody (BD Pharmingen) and a rabbit polyclonal anti-PEA-15 antibody (Abcam). Images were captured using EZ-C1 3.50 imaging software. Images were analyzed for colocalization of signals using National Institutes of Health ImageJ software.

Cell Fractionation, Immunoprecipitation and Immunoblotting

Cells were lysed in either NP40 lysis buffer (150 mM NaCl, 50 mM Tris Base pH 7.4, 1% NP40) or RIPA buffer (50mM Tris pH 7.4, 100mM NaCl, 0.1 % SDS) supplemented with complete protease inhibitor mixture (Roche), 50 mM NaF and 1 mM Na_3VO_4 and centrifuged at 13,000g for 10 min at 4°C. Protein concentration was determined by BCA assay. 500μg of protein were immunoprecipitated with 2 μg of rabbit anti-GFP antibody (Abcam) overnight at 4°C followed by 25 μl of protein A/G (Pierce). Beads were washed five times, eluted in boiling Laemmli buffer, resolved on 10% SDS-PAGE and immunoblotting was performed with mouse anti-α tubulin antibody (1:1000). The C5, C15

and DED1 specific caspase 8 antibodies described under immunofluorescence were used in immunoblotting (5μg/ml). For immunoblot analysis, 30μg of protein was boiled in Laemmli buffer and resolved on 10% gel. Centrosomes were isolated from NB7-GFP, NB7 DED-GFP stable cell lines and COS-7 cells according to the protocol of Bornens (Bornens et al., 1987). Centrosome fractions were analyzed by SDS-PAGE and Immunoblotting.

Viability analysis

Cell viability was analyzed by flow cytometry following propidium iodide staining, essentially as described (Hetz et al., 2002). Briefly, cells were harvested, resuspended in ice cold PBS containing 10 μg/ml of PI, and analyzed by flow cytometry. Apoptosis was determined by gating PI fluorescence versus Forward scatter using Cell Quest software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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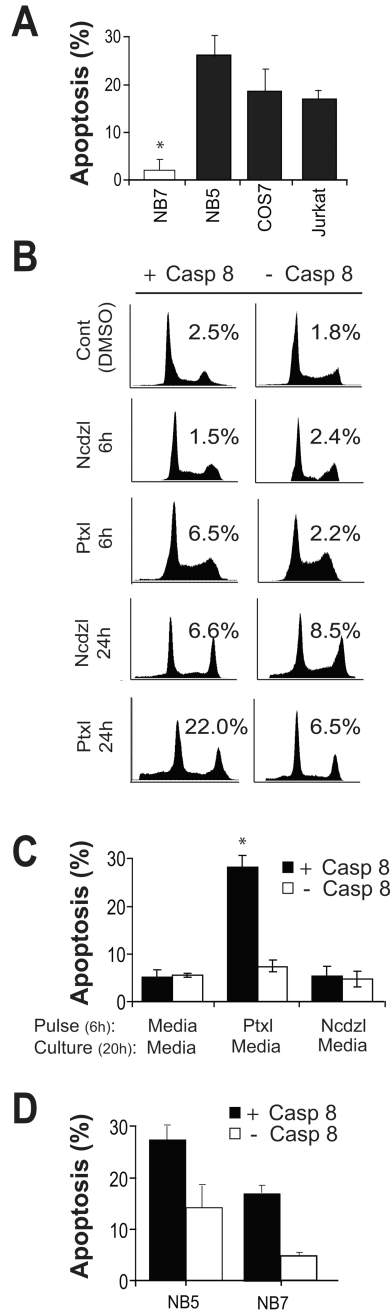
Figure 1. Caspase 8 promotes paclitaxel-mediated Apoptosis
A. Cells lacking caspase 8 (open bars) or expressing caspase 8 (filled bars), including NB7, NB5, COS-7 and Jurkat, were treated with 400nM paclitaxel for 20 hours and cell death assessed by FACS Analysis. **B.** Jurkat cells expressing or lacking caspase 8 were treated with 500nM nocodazole or 400nM paclitaxel for 6 and 24 hours and cell death assessed by FACS analysis before G2/M arrest occurred. **C.** Jurkat cells expressing or lacking caspase 8 were treated for 6 hours with 500nM nocodazole or 400nM paclitaxel, washed then left in culture for 20 hours. Cell death was assessed by FACS analysis. **D.** Neuroblastoma cells

deficient for caspase 8 (NB7) were reconstituted by stable expression of caspase 8B, while NB5 cells expressing caspase 8 were transduced with lentiviral vector encoding shRNA to caspase 8 or with control sham vector. Cells were incubated for 20h with 400nM paclitaxel, and apoptosis assessed as described above.
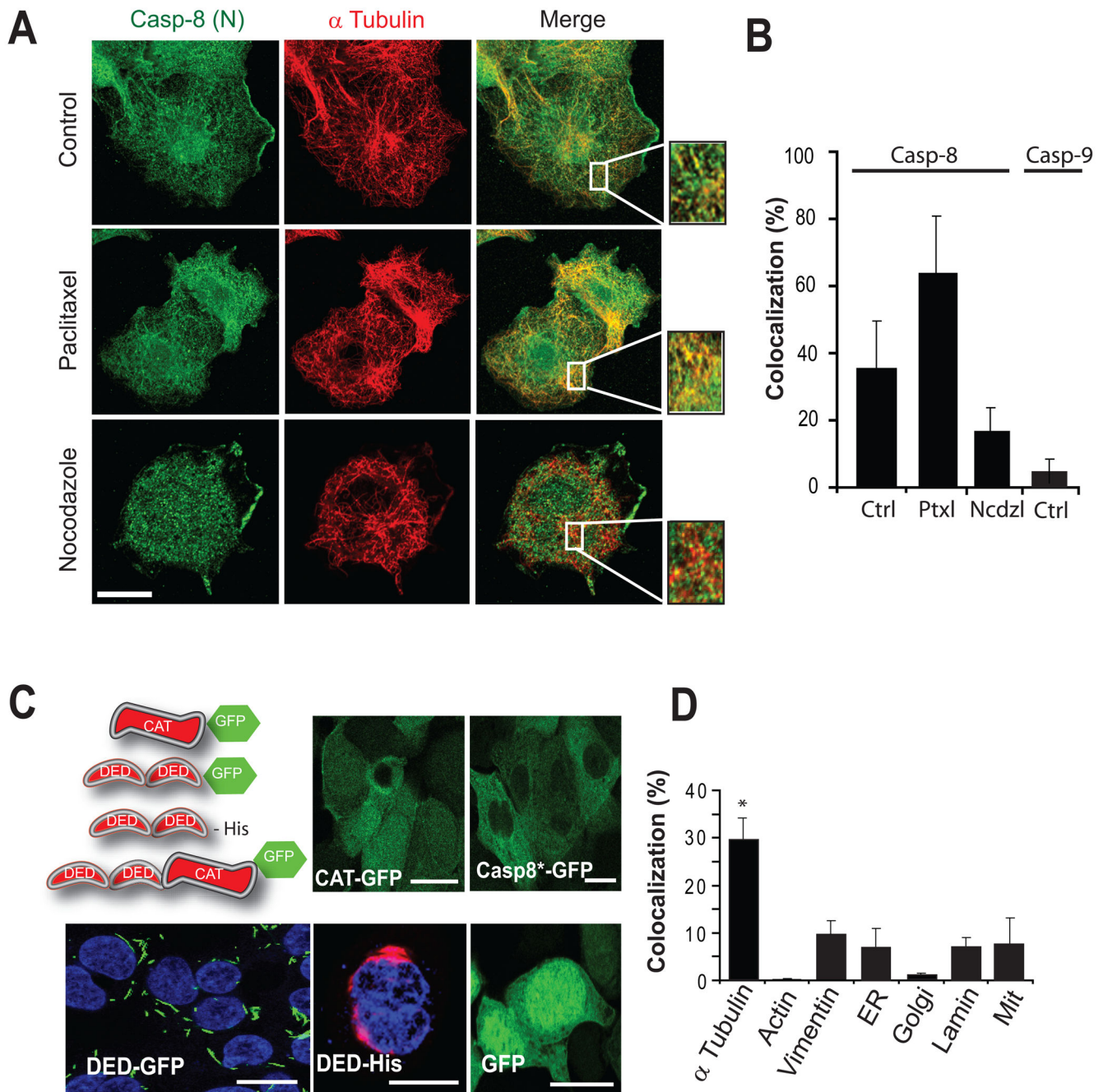
Figure 2. Caspase 8 colocalizes with microtubules

A. Confocal images of COS-7 cells in the presence of paclitaxel (400nM, 5h) or nocodazole (500nM, 5h), showing colocalization between endogenous caspase 8 (green channel) and α tubulin (red channel) (scale bar = 10μm). **B.** Colocalization of caspase 8 with microtubules increased by more than 2 fold following paclitaxel treatment (p>0.01) but appeared to decrease by more than 2 fold following nocodazole treatment (p>0.01). **C.** GFP- or His-tagged domains of caspase 8, as illustrated, were expressed in caspase-8 deficient NB7 neuroblastoma cells. Confocal images show the subcellular distribution of the inactive

catalytic domain (CAT-GFP), catalytically inactive caspase 8 holoenzyme (C8*-GFP), DED-GFP (green signal), his-tagged DEDs (red signal), and GFP when stably expressed in caspase 8- deficient neuroblastoma cells (scale bar = 10μm). **D.** Colocalization studies were performed staining cells for α-tubulin, actin, vimentin, ER, Golgi, nuclear envelope, Mitochondria (red channel) and DED-GFP (green channel). Quantification of colocalization between DED-GFP and all the cellular components described was determined using NIH ImageJ Software. Data were analyzed with ANOVA followed by Post hoc Tests (*, significant differences, p 0.05).
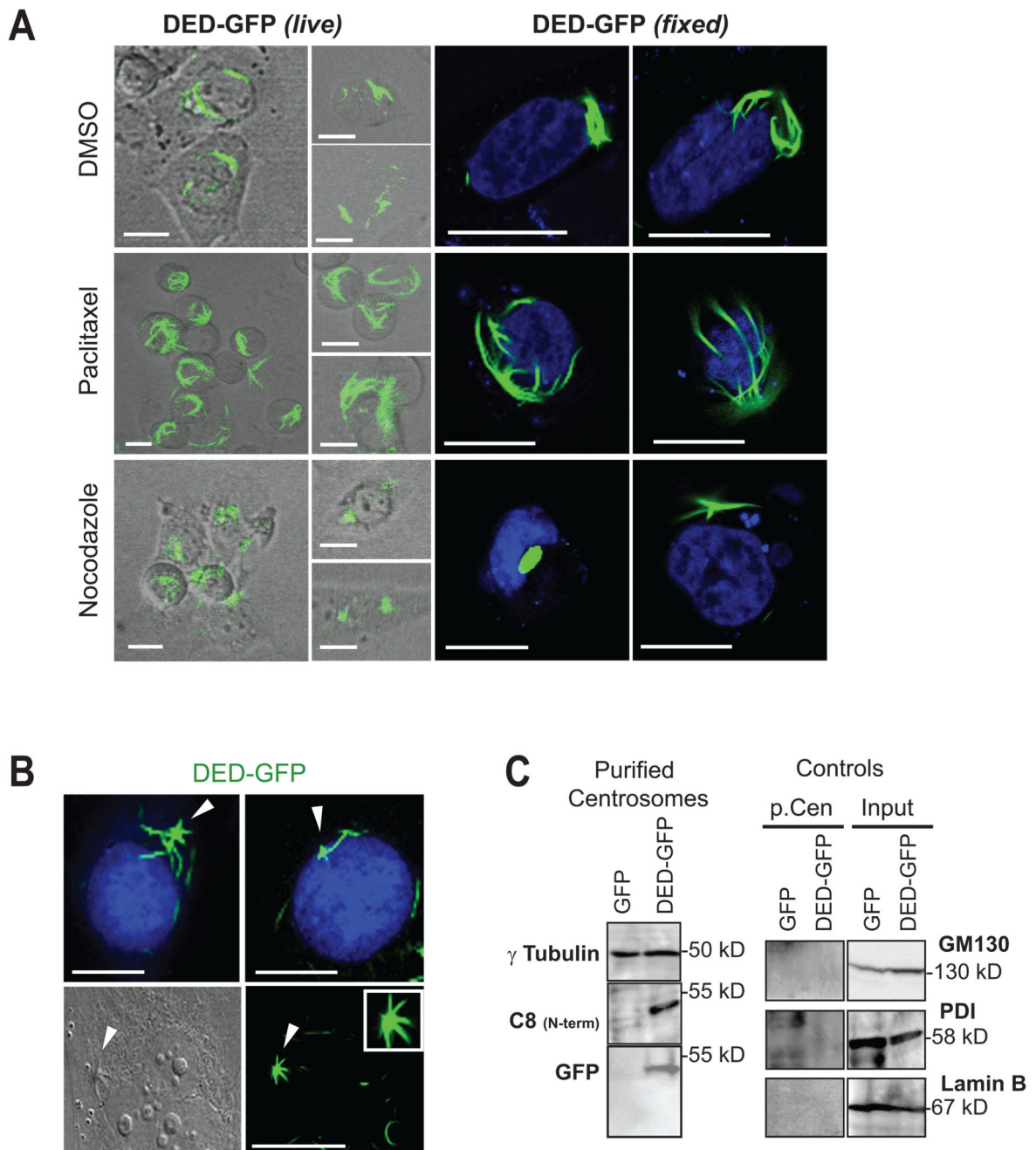
Figure 3. Caspase 8 DEDs interact with stable microtubules at centrosomes. Or Caspase 8 DEDs interact with centrosomes

A. Neuroblastoma cells expressing DED-GFP (Green channel) were treated with paclitaxel to stabilize microtubules (middle panels), or nocodazole to disrupt microtubules (lower panels) relative to DMSO controls (top panels). Localization of GFP-DEDs in living (left column) or fixed (right column) cells were assessed via confocal microscopy. Fixed cells were stained with Topro to label nuclei (Blue channel) (scale bar = 10μm). **B.** Extended confocal microscopy studies were performed staining cells for nucleus (Topro, blue channel)

and DED-GFP (green channel). DED-GFP formed an aster in the perinuclear region as shown in the paired bright field and fluorescent images. **C.** Centrosome fractions were purified from these cells by differential centrifugation (Purified Centrosomes), and probed for the presence of γ-tubulin, a centrosome marker, as well as for the caspase 8-DEDs. Purity of the preparations was assessed by immunoblot analysis of markers from potential contaminating fractions, including Golgi (GM130), endoplasmic reticulum marker (PDI), or nuclear envelope marker (Lamin B). No contamination was detected in the purified centrosome (p.Cen) preparations relative to total material (Input).
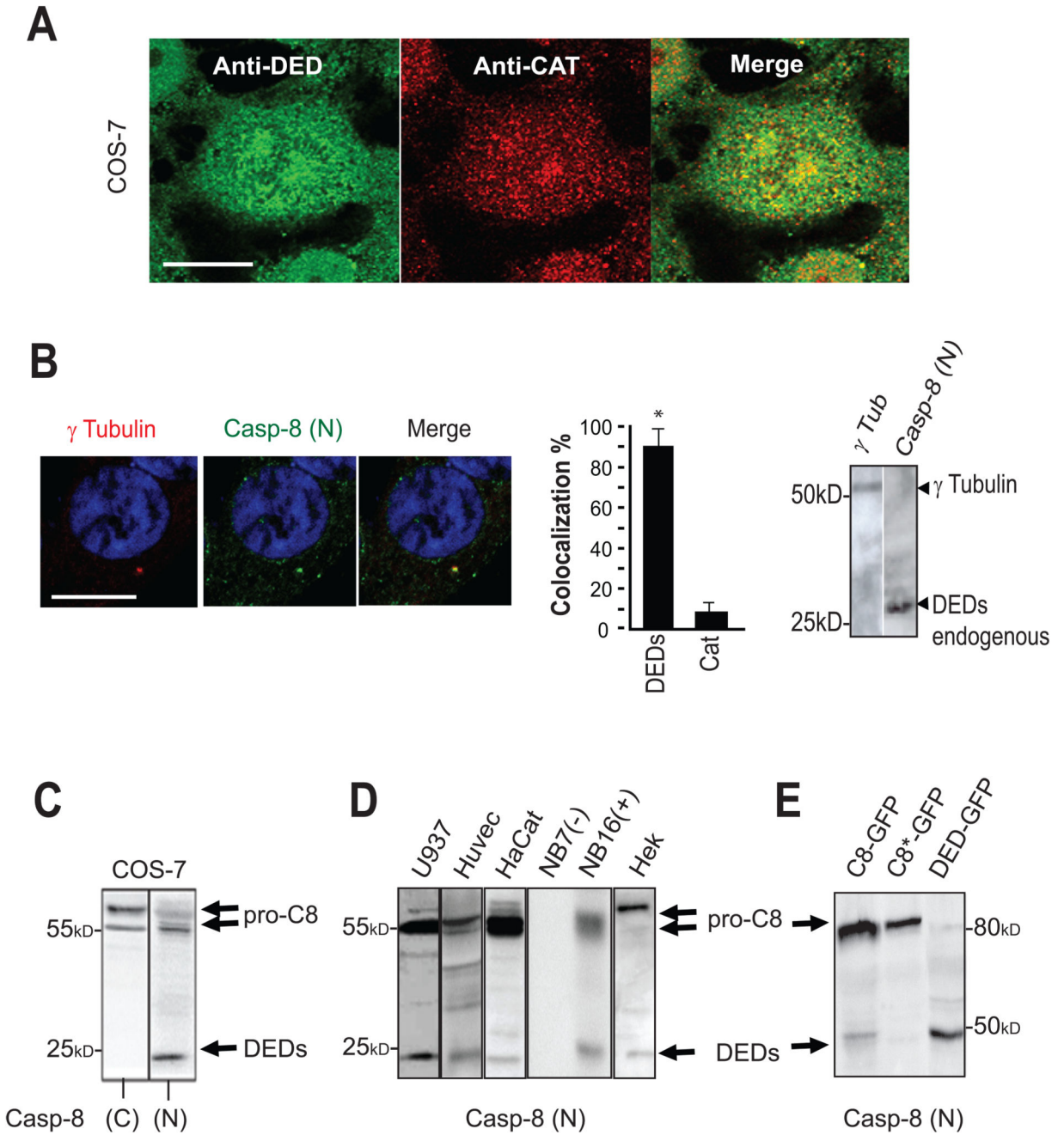
Figure 4. Expression and localization of DEDs in non-apoptotic cells
A. Confocal microscopy analysis of COS7 cells, stained with antibody to the DEDs (the N terminal domains of caspase 8, green channel), or with antisera to the catalytic domain (the C-terminal domain of caspase 8). The merge shows colocalization of the signals. **B.** Confocal microscopy images of COS-7 cells showing localization of caspase 8 DEDs (green channel) at the centrosome (red channel). The centrosome is stained with anti-γ-Tubulin (red channel), the DEDs with amino-terminal Caspase 8 antibody (green channel), and DNA/ chromosomes with Topro (blue channel) and imaged using a small confocal pinhole and

fluorescence thresholding of 80%. Microscopic quantification of cells showing colocalization of caspase 8 DEDs, caspase 8 catalytic domain. Data shown is based on a total of ~200 cells counted for each group (mean ± SD for five determinations shown). Immunoblot analysis of the centrosome fraction with Casp-8 amino-terminal specific antibodies confirmed centrosomal localization of Casp-8-DEDs on COS-7 cells, and the absence of the full length caspase 8 at this site. **C.** Immunoblot analysis of lysates from growing COS7 cells using antibodies specific to carboxy-terminal ("C," left panel only) or amino-terminal ("N," right panels) regions of caspase 8. **D.** A similarly immunoblot analysis of U937 myeloid cells, HUVECs, HaCat cells, NB7 cells (- indicates caspase 8 deficient), NB16 cells (caspase 8 expressing neuroblastoma), and 293 HEK cells is shown using the anti-DED specific antibody to detect caspase 8 (56–58kDa band) or DEDs (~23kDa, N-reactive band). **E.** The same immunoblot study was performed in NB7 cells reconstituted with Caspase 8, a catalytically inactive caspase 8 mutant (C8*), or with GFP-DEDs alone.
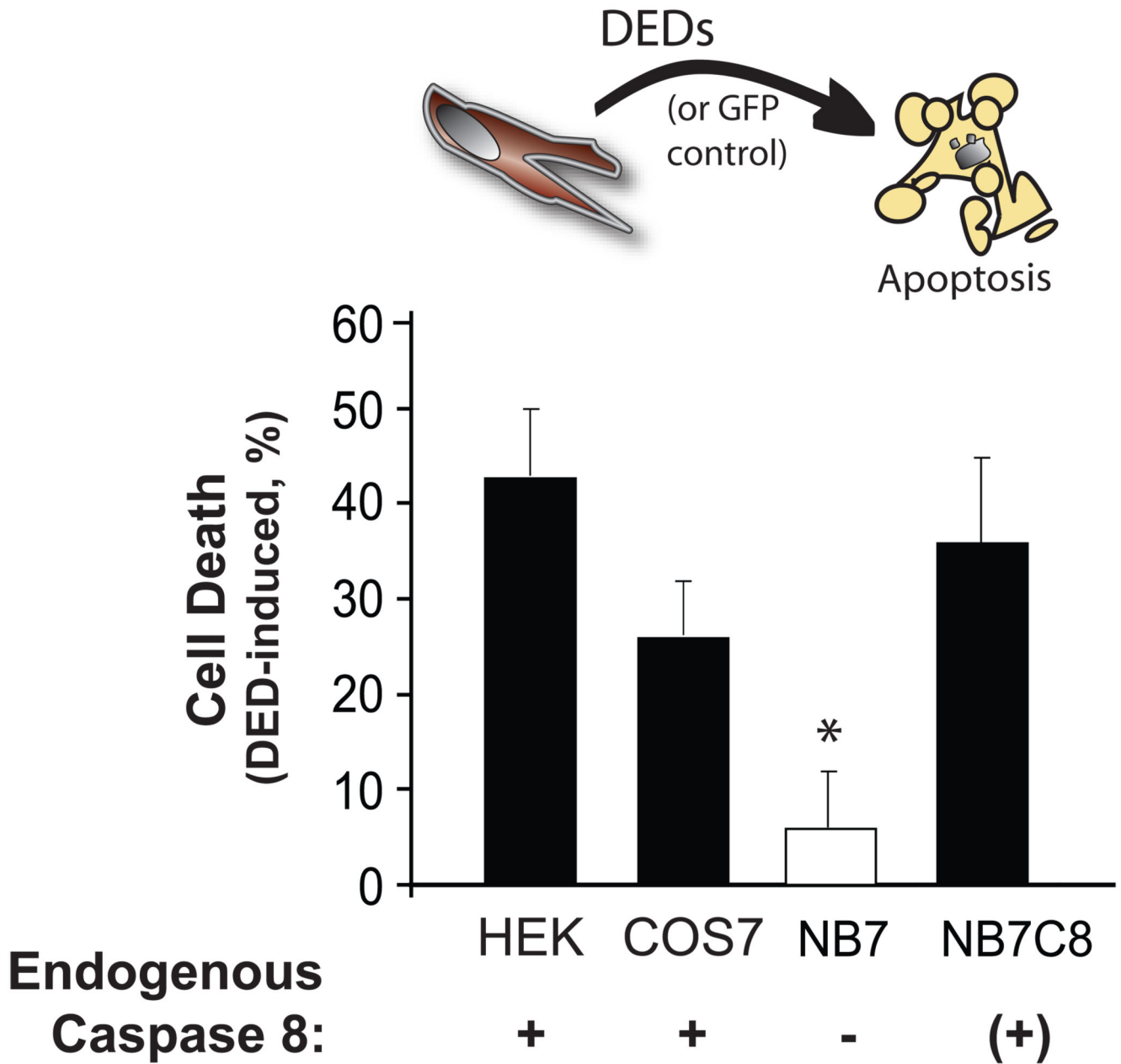
Figure 5. Expression of DEDs induces apoptosis in cells expressing caspase 8

The GFP-DED domains of caspase 8 were expressed in HEK, HaCaT, NB7 and NB7C8 cell lines for 24 hours, and the cell death induced assessed by FACS analysis of GFP-expressing cells. Caspase 8 positive cell lines have filled bars, while caspase 8 deficient cells are shown as open bars. Numbers shown represent the relative % of cell death induced above expression of GFP alone. Death was significantly less in the NB7 group than in the caspase 8 expressing cells. (* p<0.01).

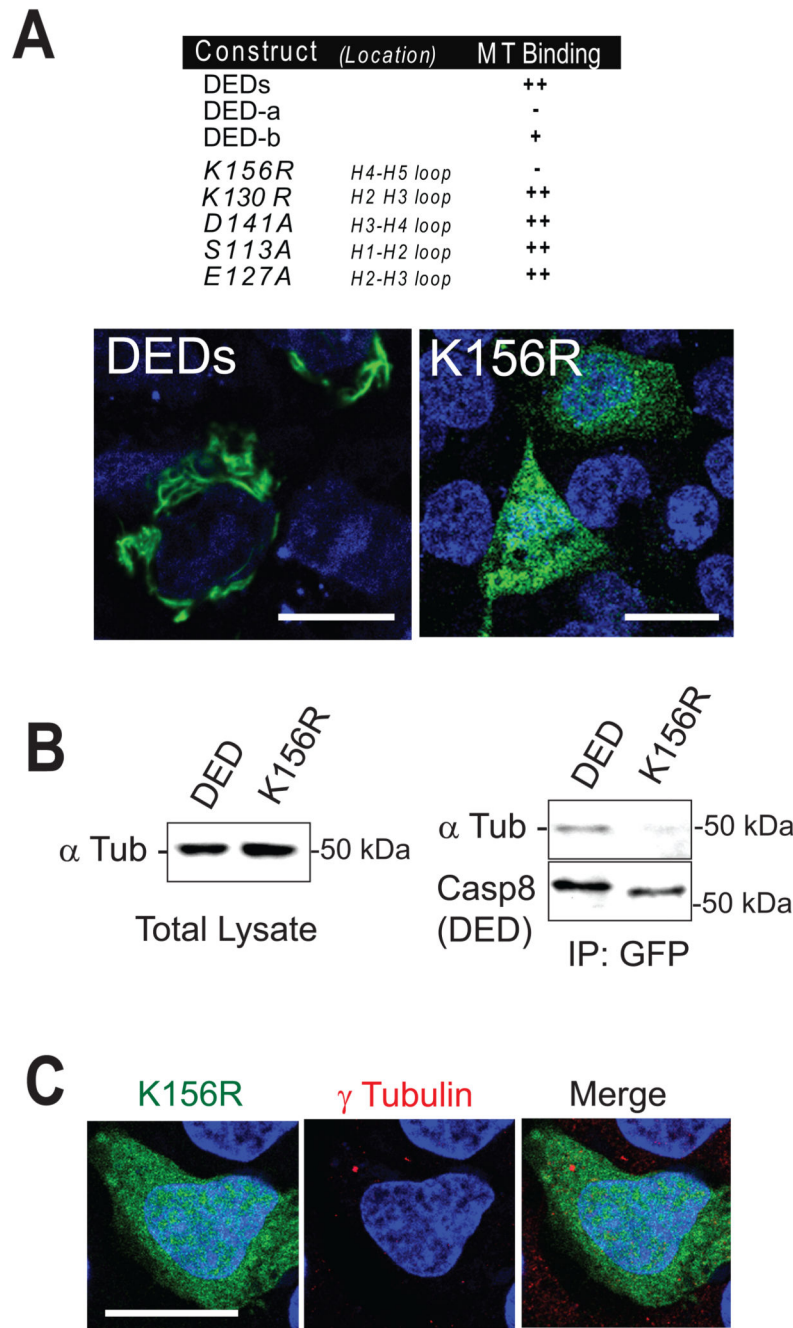**Figure 6. Requirement of Caspase 8 Lysine 156 for microtubule and centrosome binding**
A. The table shows mutants tested including those binding to microtubules behaving as wild type (wt, indicated as ++), those deficient in binding (−), and those with some binding (+); the location of each mutation in the structure of DED-b is listed; the mutations shown focus specifically on the inter-helix connecting loops. Confocal immunofluorescence images showing the distribution of wildtype DED-GFP and DED-GFP mutant Lys156Arg (K156R) when stably expressed in caspase 8-deficient neuroblastoma cells. **B.** Immunoblot analysis of α-tubulin and DED-GFP expression in GFP immunoprecipitates of wildtype DED-GFP

or DEDK156R-GFP cells. **C.** Confocal images of DED-K156R-GFP expressing cells were taken to evaluate whether this mutant (green channel) was not recruited to centrosomes (γ-tubulin, red channel) (scale bar = 10μm).
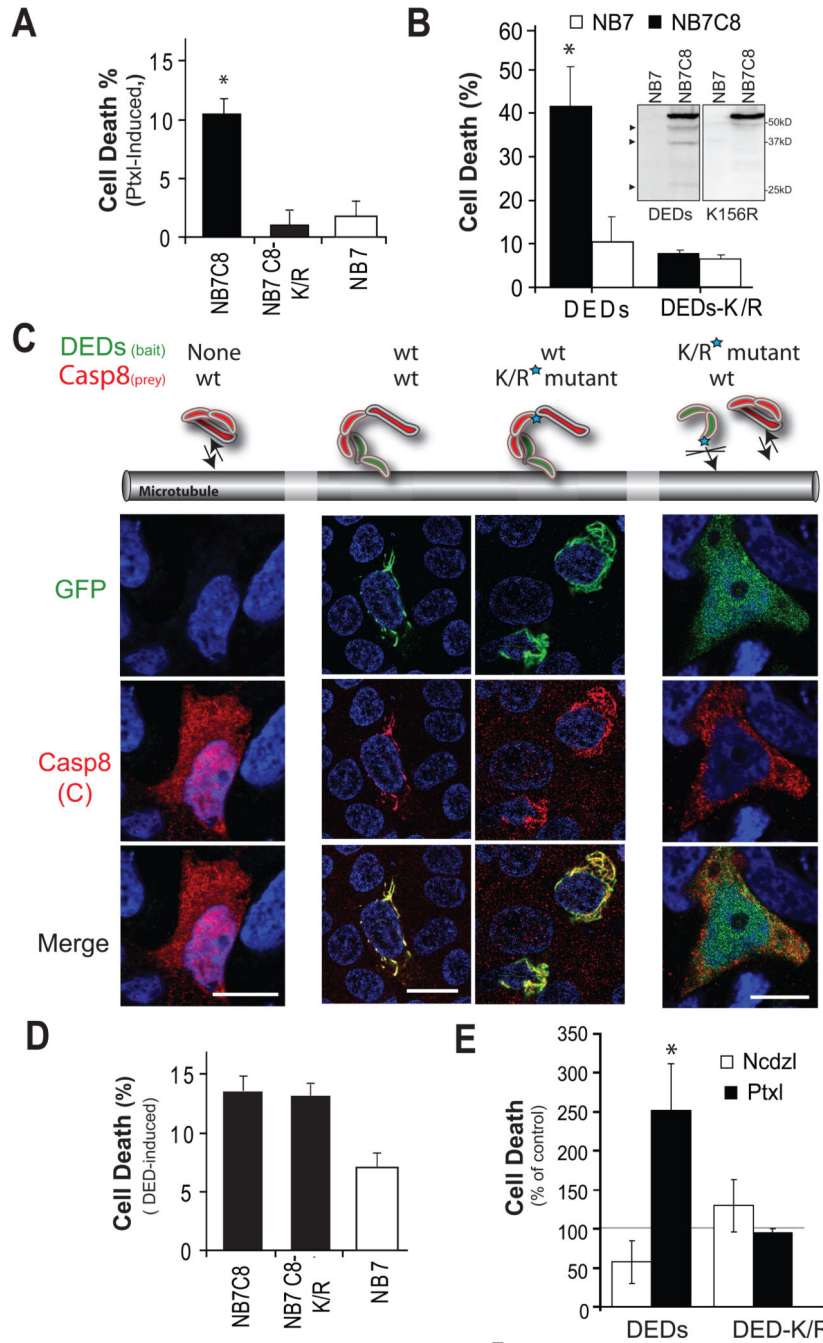
Figure 7. Recruitment to microtubules promotes caspase 8-mediated apoptosis
A. Neuroblastoma cells deficient in caspase 8 (NB7) were reconstituted with caspase 8 or the microtubule-binding mutant of caspase 8 (Casp8-K156), treated with paclitaxel (400ng/ml) and apoptosis was assessed relative to untreated controls. **B.** Quantification of apoptosis in neuroblastoma cell deficient (NB7) or expressing (NB7C8) caspase 8 following expression of wt DEDs or those deficient in microtubule binding (K156R-GFP) (10μg transfected). Caspase 8 processing during apoptosis was assessed by immunoblot analysis of these lysates using an antibody reactive to the catalytic domain of caspase 8 (inset). For all

panels, data shown are the mean ± SD from triplicate determinations. Data were analyzed with U Mann-Whitney Test (*, significant differences, p 0.05). **C.** Immunofluorescence images showing the localization of caspase 8 basally in NB7 cells (Left Panel), or following expression of DEDs (2μg transfected). Images of cells which did not appear to be undergoing apoptosis were taken (no nuclear shrinkage or blebbing). Colocalization of either Caspase 8 (Casp8) or a mutant Caspase 8 lacking the microtubule binding motif (Casp8 K156R) (red channel) following expression of GFP-labelled DEDs (green channel) was evaluated (middle panels). Both, wt caspase 8 and casp8 K156R constructs showed enhanced recruitment to microtubules by DEDs (merge, yellow channel). The right panels show expression of the K156R DEDs, which is not recruited to microtubules, and does not enhance association of wt caspase 8 with microtubules (scale bar = 10μm).