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14. ABSTRACT
This project studied a novel apoptosis pathway that is induced by the death domain of the adaptor protein FADD (FADD-DD). This pathway is important because it represents a very early defect in the development of prostate cancer and links a novel cell death pathway to the immortalization and transformation of epithelial cells. The current project is based on the finding that the tumor suppressor Bin1 can co-operate with FADD-DD to kill a prostate tumor cell line (LNCaP) that is normally resistant to FADD-DD and the grant was intended to understand the role of Bin1 in this signaling pathway. During the funding period, we achieved the goals outlined in the original grant. Moreover we were able to extend these studies in other directions and achieve goals that were not in the original grant. This work was reported in the literature and ongoing studies that are nearing completion will generate further scientific publications. In addition, based on findings supported by the award, we were able to develop a new research proposal that received RO1 funding from NCI to continue this work.

15. SUBJECT TERMS
apoptosis, tumor suppressor, cell signaling

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Introduction.

This report describes the work supported by this award, which was first activated in April 2003 when Dr. Thorburn's lab was at Wake Forest University. The lab moved in September 2004 to the University of Colorado Health Sciences Center and we received permission to transfer the grant to the University of Colorado in 2005. As outlined here and in the previous annual reports, we made good progress and achieved all the original aims despite the disruption associated with the move.

Scientific Background.

Evasion of apoptosis is a hallmark of cancer. Consequently, it is sometimes thought that cancer cells are generally resistant to apoptosis while normal cells are sensitive. In fact cancer cells are actually closer to their apoptotic threshold than their normal counterparts and therefore often undergo apoptosis more easily in response to diverse apoptotic stimuli. This apoptosis sensitization occurs because growth promoting oncogenic events raise the levels of caspases and other apoptotic proteins or make it easier to activate these molecules and thus reduce the threshold at which apoptosis is activated. However, it is not clear if this is the only apoptotic barrier that cancer cells must overcome as they become transformed. Are there also specific apoptosis pathways that inhibit cancer development and are active in normal cells and specifically inactivated during tumor development? We hypothesized that such a pathway would have the unusual characteristic of working in normal cells but not in cancer cells.

The cell death pathway that we study is induced by a protein interaction domain from an adaptor protein called FADD-DD and works in normal epithelial cells but does not work in immortalized epithelial cells. Moreover this cell death pathway has other very unusual characteristics because it involves both caspase-dependent apoptosis and another form of cell death that during the course of this project we identified as autophagy. Tumor cells are not normally sensitive to FADD-DD-induced cell death however, we discovered that a prostate cancer cell line (LNCaP) could become sensitive if we express the Bin1 tumor suppressor. This project was designed to further investigate this response by determining whether the same cell death pathway is activated by FADD-DD +Bin1 in the cancer cells as is activated by FADD-DD alone in normal cells, determining which parts of Bin1 are important for this response and testing whether Bin1 is essential for the response to FADD-DD in normal cells.

Body.

As described in our previous annual reports and the report that was submitted when we transferred the grant to the University of Colorado, we achieved the goals outlined in the original grant and statement of work. In our most recent annual report, we discussed experiments showing that the ability of FADD-DD to co-operate with Bin1 is selective for LNCaP cells, we showed that caspases are activated in LNCaP cells when FADD-DD is combined with wildtype Bin1 but not with a mutant Bin1 molecule that lacks the domain identified in the first year as being important and showed that this effect is specific because Bin1 does not sensitize LNCaP cells to other apoptotic stimuli. In addition, we discussed the paper showing that autophagy is involved in the FADD-DD

death mechanism and discussed the data (described in the same paper) showing that TRAIL, a physiological stimulus can activate the same pathway. The only remaining question from our original grant was to determine whether interference with Bin1 could alter the FADD-DD-induced cell death in normal prostate epithelial cells (task 3 in the original Statement of Work). This was the most important question in the grant and also the most difficult to address because it requires that we find a way to selectively inactivate Bin1 in normal prostate epithelial cells and then ask how this alters the response of those cells to FADD-DD. As proposed in our most recent annual report, based on new studies that we performed during this project, we first tried to test the hypothesis using conditional knockout of the Bin1 gene in primary prostate epithelial cells from the "floxed" Bin 1 mouse. Unfortunately these experiments proved problematic because we could not reliably knockout the Bin1 gene using adenovirus – mediated expression of Cre recombinase in the primary epithelial cells from these animals. We therefore refocused out efforts on human cells. We had to develop new methods to perform reliable siRNA knockdown in the primary prostate epithelial cells. Fig. 1 shows that this was successful and we could selectively remove Bin1 from primary human prostate epithelial cells.

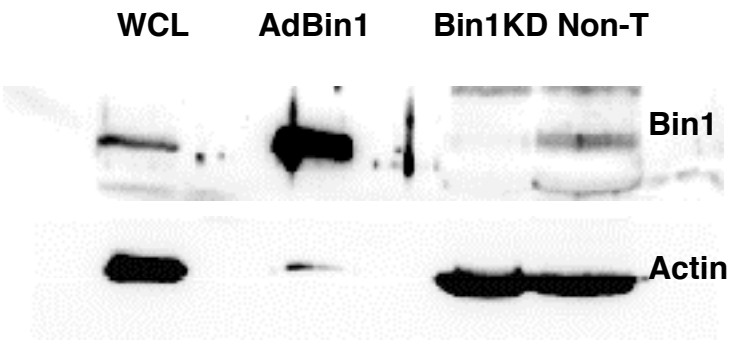

Fig. 1. siRNA knockdown of Bin1 in normal prostate cells. Prostate epithelial cells were transftece with Bin1 siRNA or a non-targeting control for 4 days. Western analysis indicates that the endogenous Bin1 protein which runs at the same size as the adenovirus induced cotnrol protein was selectively depleted in Bin1 siRNA transfected cells.

We next asked if Bin1 depletion prevented FADD-DD-induced death or changed the death. In a previous paper (Thorburn et al., 2003), we showed that FADD-DD-induced death of normal primary prostate epithelial cells can only be blocked by combining a caspase inhibitor with a serine protease inhibitor. Therefore, if our central hypothesis is correct and Bin1 is involved in the FADD-DD death pathway in normal prostate epithelial cells, we should find either that Bin1 knockdown would make primary prostate cells less sensitive to FADD-DD-induced death and/or would alter the way that the cells die as shown by altered sensitivity to caspase inhibition or serine protease inhibition. Figure 2 shows that this was indeed the case therefore suggesting that the hypothesis is correct. Bin1 knockdown did not significantly alter the overall amount of death caused by FADD-DD but unlike the case in control cells where Bin1 was not knocked down (see our previous paper (Thorburn et al., 2003) for more discussion), caspase inhibition alone was able to significantly inhibit the death. These data show that Bin1 is involved in the FADD-DD pathway in normal prostate cells and therefore answer the remaining questions that were proposed in our original grant and statement of work.

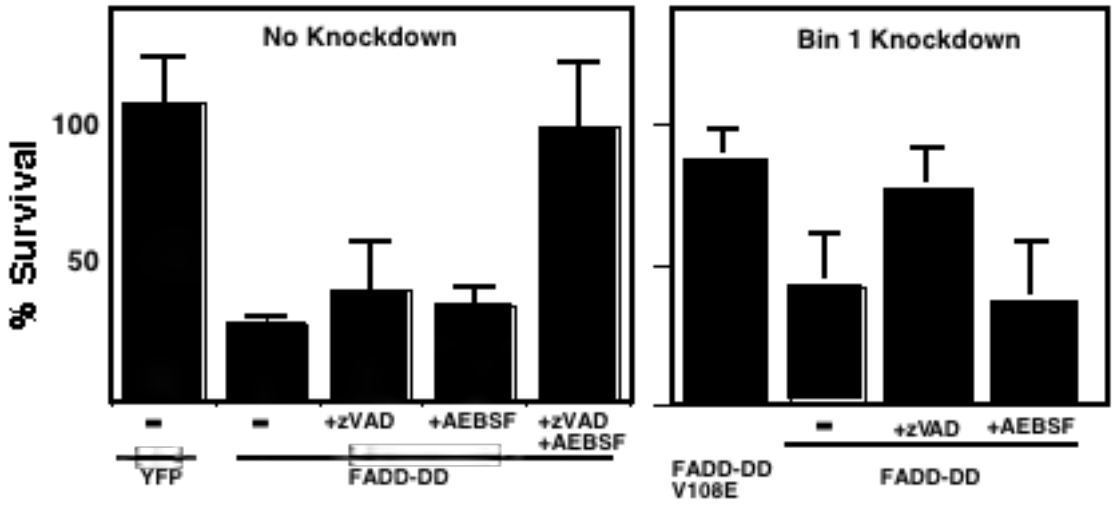

Fig. 2. Bin1 knockdown alters the way that FADD-DD kills normal prostate epithelial cells. Normal human prostate epithelial cells were injected with FADD-DD, YFP control or the inactive FADD-DD mutant V108E as indicated after no further treatment (left panel) or transfection with Bin1 siRNA (right panel). As previously reported, FADD-DD-induced death is blocked only when both zVAD and the serine protease inhibitor AEBSF are used at the same time. However when Bin1 is knocked down, zVAD alone can provide protection.

During the course of our work (and discussed in previous annual reports), we found that TRAIL a physiological activator of FADD signaling could activate the FADD-DD death pathway that involves both caspases and AEBSF-sensitive serine proteases (Thorburn et al., 2005). Therefore if the Bin1/FADD-DD pathway that we have been studying in LNCaP cells is working in the same way, we would expect that when we have Bin1 present in LNCaP cells, we would lose the ability of caspase inhibitor zVAD to completely block TRAIL-induced death. Moreover if these ideas are correct, the Bin1 mutant that lacks the domain required for FADD-DD cooperation (discussed in previous annual reports) should not alter the ability of zVAD to block TRAIL-induced death. We therefore performed experiments to test this hypothesis. Fig. 3 shows that as expected TRAIL could kill LNCaP cells but when wildtype Bin1 is present zVAD can only partially block this death. However in the presence of the Bin1 mutant 10d4, zVAD completely blocks TRAIL-induced death. These studies on TRAIL have been furthered by detailed analysis of how FADD interacts with and is activated by TRAIL receptors described in several recent papers that were also carried out during this grant, (Thomas et al., 2006; Thomas et al., 2004a; Thomas et al., 2004b).

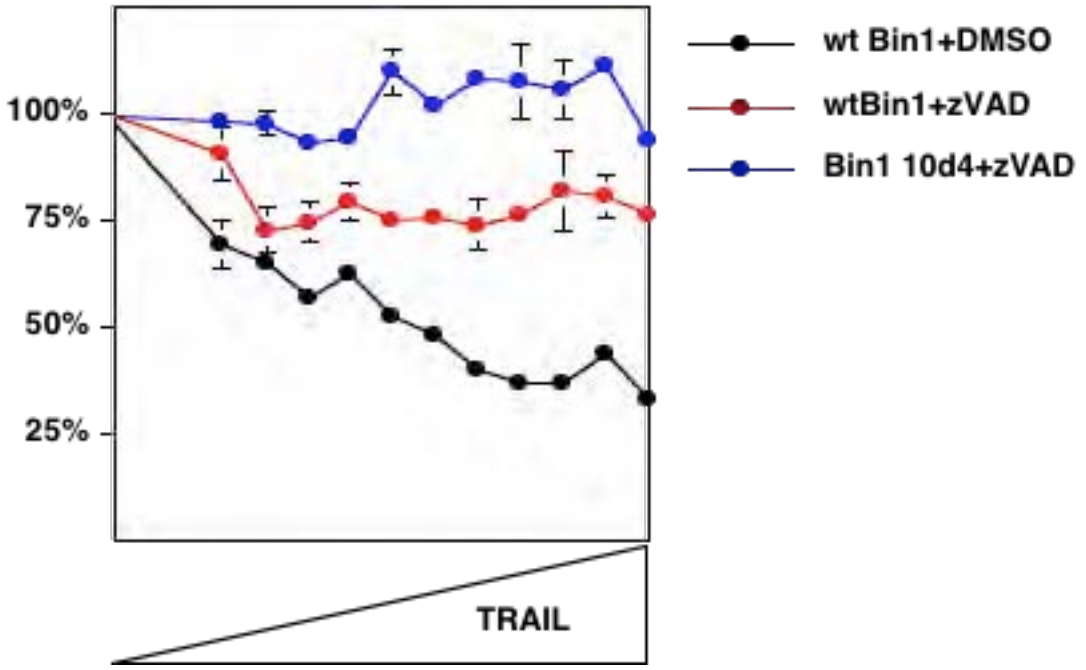

Fig. 3. Bin1 expression alters the sensitivity of TRAIL-induced death in LNCaPs to caspase inhibition. LNCaP cells were infected with wildtype or mutant Bin1 and treated with increasing concentrations of TRAIL in the presence or absence of the caspase inhibitor zVAD as indicated. Note that zVAD cannot completely block TRAIL-induced death wildtype Bin1 expressing cells.

Key research accomplishments.

- We achieved the goals set out in the original grant and were able to extend our studies to make inroads into other areas including the role of TRAIL receptor signaling in this response and the role of non-apoptotic cell death via autophagy.
- We showed that LNCaP cell death induced by the combination of FADD-DD and Bin1 can be inhibited only when caspases and serine proteases are inhibited. Neither class of inhibitor on its own prevented death.
- We showed that caspase activity is induced by Bin1 plus FADD-DD in LNCaP cells. Caspase activity is not induced by Bin1 alone, FADD-DD alone or Bin1 plus a FADD-DD point mutant that is inactive.
- We identified the Bin1 BAR domain as being required for co-operation with FADD-DD and showed that point mutants in this domain abolish co-operation.
- We demonstrated that Bin1 co-operates with FADD-DD to kill LNCaP cells via a mechanism that involves both apoptosis and another form of cell death. As we originally hypothesized, this finding further suggests that Bin1 allows FADD-DD to activate the same cell death pathway that we identified in normal prostate cells.
- We demonstrated that other prostate tumor cells (e.g. DU-145) do not display co-operation between FADD-DD and Bin1 demonstrating that prostate tumor cells can adopt other strategies to subvert this cell death pathway in addition to loss of Bin1.
- We demonstrated that Bin1 does not sensitize LNCaP cells to other cell death stimuli. Together with our previous work, this result further shows that the co-

operation with FADD-DD is specific and suggests that Bin1 is directly involved in the FADD-DD pathway supporting our overall hypothesis for this project.

- We demonstrated that the alternate form of cell death that occurs in addition to caspase-dependent apoptosis is autophagy. This provides new insights into the death pathway and the first link between autophagy and prostate cancer development.
- We developed methods to perform reliable siRNA knockdown experiments in normal, primary human prostate cells and showed that Bin1 knockdown alters the death pathway induced by FADD-DD. This shows that Bin1 is involved in the pathway in normal cells too.
- We showed that a physiological activator of FADD signaling (TRAIL receptor activation) couls activate the same signaling pathway and that the presence of absence of Bin1 alters the response of LNCaP cells to TRAIL pathway activation. This suggests that physiological activation of endogenous FADD activates the same Bin1-dependent signaling pathway.
- These data provide strong support for the central hypothesis that was described in the original grant- i.e. that bin1 is an integral part of the novel FADD-DD death pathway that is selectively inactivated during the earliest steps of prostate cancer development.

Reportable outcomes.

Publications.

Thorburn, A. (2004). "Death receptor-induced cell killing." *Cellular Signalling*. **16,** 139-144.

Thorburn, J., Moore, F., Rao, A, Barclay, W., Thomas, L.R., Grant, K.W, Cramer, S.D. and Thorburn, A. (2005) "Selective inactivation of a FADD-dependent apoptosis and autophagy pathway in immortal epithelial cells." *Mol. Biol. Of the Cell*. **16,** 1189-1199.

We are currently working on a further manuscript describing the as yet unpublished work on the relationship of Bin1 and FADD-DD. Data that will be reported in this paper is in this report and our previous annual reports.

Grants.

Based on the work that was supported by this grant, we were able to develop a new 5 year NIH grant (CA111421) that was funded in 2005 and will run through 2010. This grant will extend the studies described here.

Conclusions.

We achieved the goals outlined in our original grant and also managed to develop new research directions. Although not all of this work is published yet, we have published some of the key findings and are working on putting together a major manuscript that will describe the as yet unpublished data (which was described in this and previous annual reports). We also obtained NCI funding to extend and continue this work. In addition, we made numerous other observations. One of these was the identification of autophagy as a process that is involved in the caspase-independent death mechanism that occurs in response to FADD-DD. This work (which was described in a 2005 paper) is, we believe, the first connection between prostate cancer development and autophagy. Therefore we believe that this grant has been very successful in achieving its goals.

What does this mean for prostate cancer?

Because the FADD-DD pathway is selectively inactivated at an early step in prostate cancer development, it may represent an early link between cell growth disregulation and apoptosis regulation that is important for cancer development. The studies supported by this grant show that this pathway involves the tumor suppressor Bin1 and thus provide new insights into why Bin1 loss, which is known to occur in prostate cancer, promotes development of the disease. Further understanding of these mechanisms may therefore identify new therapeutic targets for manipulating this pathway. If we can find ways to do this, we may be able to re-activate this apoptotic pathway in prostate cancer cells, which should result in tumor cell-specific cell killing that could be a useful treatment to limit the development of the disease.

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Review article

Death receptor-induced cell killing

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Abstract

Apoptosis pathways activated by death receptors of the tumour necrosis factor (TNF) family such as Fas, TNFR1, or the TRAIL receptors DR4 and DR5 are implicated in diverse diseases. These are also the best-understood apoptosis pathways and many of our ideas about apoptosis regulation come from studying these pathways. Cell killing from such receptors occurs because of recruitment to the receptor of the adaptor protein FADD, which in turn recruits the pro form of caspase-8. Aggregation of pro-caspase-8 leads to its auto-activation and subsequent activation of effector caspases such as caspase-3. The apoptotic signal can be amplified through the mitochondria and inhibited through the action of competing molecules such as the inhibitor c-FLIP, which binds to the receptor complex in place of caspase-8. This simple mechanism explains much of the cell death that is induced by death receptors. However, recent studies indicate that we must incorporate new information into this model. Some examples that add new layers of complexity will be discussed in this review.

Keywords: Death receptors; TNF; Caspase-8

1. Introduction

Two main pathways that activate caspases, the proteases that are the primary drivers of apoptosis, have been identified. Diverse stress pathways cause release of mitochondrial proteins to activate the "intrinsic pathway" [1]. Mitochondrial protein release often occurs after BH-3-only members of the Bcl-2 family [2,3] bind to and neutralize anti-apoptotic members of the Bcl-2 family. This promotes release of mitochondrial proteins including cytochrome *c* through an as yet incompletely characterized mechanism. Released cytochrome *c* interacts with Apaf-1, pro-caspase 9 and dATP to form a complex called the apoptosome [4]. This complex dimerizes and activates caspase 9, which then activates effector caspases to induce apoptosis. Other released mitochondrial proteins that promote apoptosis include Apoptosis Inducing Factor (AIF) [5], Smac/Diablo [6,7], Endonuclease G [8] and Omi/HtrA2 [9–12]. Recent work suggests that these mitochondrial effects might occur downstream of other caspases, particularly caspase-2 [13–15] and that Bcl-2 proteins might function at this stage too, i.e. before any mitochondrial activity [3,15].

The other apoptotic pathway, the "extrinsic pathway", which is activated by ligand-bound death receptors such as tumour necrosis factor (TNF), Fas or TRAIL receptors, has been thought to be much simpler and better understood [16]. Although death receptors can promote cell growth under at least some situations [17], the ability of these receptors to induce apoptosis is critical in several disease processes and has been the focus of most work to date. The six known death receptors contain an intracellular globular protein interaction domain called a death domain (DD). Upon ligand binding to death receptors probably in the form of pre-associated receptor trimers [18,19], activated death receptors recruit an adaptor protein called Fas Associated Death Domain (FADD) [20]. FADD consists of two protein interaction domains: a death domain and a death effector domain (DED). The current model is that FADD binds (directly or via another adaptor such as TRADD, which binds to TNFR1) to the receptor through interactions between DDs and to pro-caspase-8 through DED interactions to form a complex at the receptor called the Death Inducing Signalling Complex (DISC). Recruitment of caspase-8 through FADD leads to its auto-cleavage and activation [21]. Active caspase-8 in turn activates effector caspases such as caspase-3 causing the cell to undergo apoptosis by digesting upwards of a hundred or so proteins [22]. An endogenous inhibitor, c-FLIP, which is related to caspase-

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8 but has no protease activity, is thought to function by competing with caspase-8 for binding to the DISC [23,24]. The BH3-only protein Bid is cleaved by caspase-8 and is then translocated to the mitochondria to activate the intrinsic pathway [25], thus connecting the two caspase activation pathways and amplifying the death receptor apoptotic signal. Thus, the current view of the mechanism of death receptor-induced apoptosis involves the formation of protein complexes that lead to activation of caspases and amplification of the death signal through the mitochondrial apoptosis pathway. Some recent findings that add new levels of complexity to this model will be discussed in this review.

2. Do death domains and death effector domains bind independently to their targets?

The current model is that FADD interacts with the receptor (e.g. in the case of Fas) or another adaptor (e.g. TRADD in the case of TNFR1) through its DD. At the same time, the other interaction domain in FADD, the DED, interacts with a DED on caspase-8. This model implies that the DD and DED function independently of each other and that these domains and the related CARD domain, which have similar structures made up of six alpha helices [26], homodimerize but do not heterodimerize. That is, a DD will interact with another DD and a DED will interact with another DED but the DED is not involved in the DD interaction and vice versa. Evidence for this idea comes from the fact that the isolated FADD DD can bind to Fas or TRADD. Indeed, this is the mechanism by which the isolated FADD DD functions as a dominant negative inhibitor of Fas-, TNFα- or TRAIL-induced apoptosis [27]. Solution structures of the isolated FADD and Fas DDs support this model and suggest that interaction takes place on a charged surface patch [28,29]. Targeted mutagenesis experiments further suggest that the same surface is involved in the FADD–TRADD interaction [30]. It was therefore surprising when a random genetic screen to identify point mutants in the FADD cDNA that prevent binding to Fas identified a series of mutations that affected the DED, not the DD [31]. The experiment used a modified method of reverse two-hybrid screening to identify point mutants in FADD that cannot interact with the Fas DD. As the name suggests, reverse two hybrids screen for loss of protein–protein interactions. A library of random mutants is generated and those that cannot interact with a target protein identified. This approach often identifies uninformative mutants that have severely disrupted protein structure or altered protein stability. In the modified method, such mutants are avoided by simultaneously screening for retention of interaction with a different protein (in this case TRADD).

According to the current model, a screen for FADD mutants that cannot interact with the DD of Fas but can interact with the DD of TRADD should identify mutations in the DD of FADD. Some mutants in the FADD DD with these characteristics have been identified [31] (L. Thomas and A.T., unpublished observations). However, the majority of the mutants that were identified in the reverse two-hybrid screen for FADD mutants that cannot interact with Fas were in the FADD DED. Specifically, mutations of amino acids in the loops that flank helix five of the DED abolished interaction with Fas but had no effect on the TRADD–FADD interaction. When these mutants were expressed in FADD-deficient cells, they rescued TNF-induced signalling (which requires TRADD binding) but could not rescue Fas ligand-induced signalling. This suggests that the binding specificity in mammalian cells is the same as in yeast. The simplest interpretation of these data is that both the DED and DD of FADD participate in the interaction with death receptors such as Fas and that the current model whereby different domains (i.e. DDs, DEDs and CARD domains) function independently of each other are oversimplified. A direct test of this idea will require structural information of the intact proteins rather than the isolated domains that have been used for previous structural studies. Because the mutations in the DED affect Fas but not TRADD binding, these data suggest that specificity of interactions between FADD and death receptors can be achieved through regulating the structure of the DED. This represents a new mechanism by which regulation could be imposed on these systems and could be used by cellular proteins to allow regulated activation of FADD by some receptors but not others. In addition, practical benefits might be obtained based on these ideas. For example, it may be possible to identify small molecules that alter the DED structure in a way that would inhibit Fas-induced apoptosis without affecting TNFR1 signalling. In recent work, we (L. Thomas et.al., manuscript in preparation) have identified a similar role for the FADD DED in the recruitment to the activated TRAIL receptors DR4 and DR5 indicating that other receptors also regulate DISC formation through the FADD DED.

3. How are caspases activated by death receptors?

The above discussion suggests that the binding of the adaptor that brings pro-caspase-8 to the activated death receptor complex is more complicated than previously thought. Other studies suggest that the next step is also not quite as simple as we previously thought. It was initially thought that pro-caspase-8 was brought to the receptor complex by FADD and that this resulted in self-activation of the caspase through a cross-proteolysis mechanism. That is, two pro-caspase-8 molecules were brought in close proximity and one molecule could digest the other molecule resulting in the formation of active, processed caspase-8. However, recent data indicate that caspase-8 activation occurs as a result of dimerization rather than processing per se and that the initial processing steps arise through intramolecular digestion rather than intermolecular digestion. Support for this model comes from experiments showing that regulated dimerization of a wildtype procaspase-

8 molecule and a mutant that does not have a functional active site can induce catalytic activity [32].

Interestingly, naturally occurring molecules that are effectively catalytically inactive pro-caspase-8 molecules are important regulators of death receptor-induced apoptosis. These are the cellular FLICE-like Inhibitory Protein (c-FLIP) proteins [24,33]. c-FLIP comes in two main isoforms, cFLIP-long and c-FLIP-short. Both proteins possess two DED motifs that are very similar to the DED's on pro-caspase-8. The short c-FLIP isoform consists of only these DED domains. However, the long protein also has a domain that is homologous to the catalytic domain of caspase-8. The long form of c-FLIP is not an active protease because it has several alterations that affect the active site of the enzyme. Both c-FLIP proteins and several viral proteins that are homologous to FLIP-short were originally thought to be solely inhibitors of death receptor-induced apoptosis that function by competing with pro-caspase-8 for binding to FADD at the activated DISC. In support of this view, increased levels of c-FLIP are associated with cancer and can confer protection against Fas ligand- or TRAIL-induced apoptosis [24,33]. However, recent work shows that the long form of FLIP when recruited to the DISC can actually promote caspase-8 activation [34,35]. This occurs because the FLIP-L and procaspase-8 dimerize and this activates the proteolytic activity of the caspase-8 molecule. One conclusion from this work is that caspase-8 activation can occur even in the absence of caspase-8 processing, thus supporting the conclusions arising from studies of artificially dimerized caspase-8 molecules. These data suggest that FLIP-L and FLIP-S are not equal in their ability to inhibit death receptor-induced apoptosis and indeed FLIP-L may promote apoptosis at least when expressed at certain levels. These findings could have important consequences for understanding cellular responses to various signalling events. For example, protein synthesis inhibitors can promote apoptosis induced by death receptors because they reduce FLIP levels [36]. Subtle differences in the relative levels of FLIP-L, FLIP-S and procaspase-8 might activate or inhibit caspase activity depending on the particular stoichiometry of these molecules at the DISC.

Detailed analysis of the mechanism of activation of the wildtype procaspase-8 protein (i.e. without artificial dimerization domains) supports the above studies. Using biochemical and biophysical approaches, it was demonstrated that dimerization is required and sufficient for caspase-8 activation but that caspase-8 processing is neither necessary nor sufficient for catalytic activity [37,38]. Rather than being required for activity, cleavage appears merely to stabilize the caspase-8 dimer. The fully processed, stable caspase-8 dimer may also have a different substrate specificity in vivo compared with the unprocessed or partly processed, active caspase-8 dimer. The idea is that active, dimerized but incompletely processed caspase-8 may remain bound to FADD at the DISC. Such a caspase-8 molecule could only digest potential substrates that are also localized in the proximity of the DISC. Important caspase-8 substrates are indeed found at the DISC including the protein kinase RIP [39] and components of the actin cytoskeleton, which must be digested by caspase-8 to promote Death Receptor internalization [40]. However, many other important caspase-8 substrates including pro-caspase-3 and Bid are less likely to be in close proximity to the DISC. Therefore while dimerization causes activation of caspase-8, this in itself may not be sufficient to allow all potential caspase-8 substrates in the cell to be digested. Because a procaspase-8-FLIP-L dimer can be catalytically active but is not completely processed, this molecule might also remain anchored at the DISC and thus be available to digest substrates that are also at this site.

An important practical consequence of these findings is that identification of the processed forms of caspase-8, which has been widely used as an assay for caspase-8 activation, is probably no longer valid. Instead, it should be borne in mind that the presence of non-processed but activated caspase-8 may lead to the cleavage of a subset of potential substrates, which could in turn stimulate only some of the cellular responses associated with apoptosis. It will be interesting to determine if different substrates are indeed cleaved in vivo by partially processed, compared with fully processed caspase-8 and to determine how this affects cellular responses.

4. Do these interactions always occur at the cell membrane?

Fas and TRAIL receptor DISC immunoprecipitations show that a stable ligand–receptor complex that contains FADD and caspase-8 occurs after receptor activation and it is therefore thought that the initial caspase activation events that lead to receptor-induced cell death occur at the cell membrane. However, important pro-apoptotic signalling events involving these proteins may also occur at other locations in the cell. In the case of the TNFR1 apoptotic signal, it was recently shown that while TRADD, RIP and TRAF2 were bound to the receptor, FADD and caspase-8 were not detectable in the complex. Nevertheless, the authors concluded that the apoptotic signal required FADD and caspase-8 and suggested that the activation of these molecules must occur somewhere other than at the membrane-bound receptor [41]. If this idea is correct, then we might expect to find TRADD, FADD, etc., at other locations in the cell. Recent studies found that this is in fact the case. TRADD was shown to have nuclear import and export sequences that cause rapid shuttling between the cytoplasm and the nucleus and inhibition of nuclear export with leptomycin B causes accumulation of TRADD in nuclear structures that are associated with promyelocytic leukemia protein (PML) nuclear bodies [42]. The nuclear accumulation of TRADD is tightly regulated but the precise details of this regulation and the physiological signals that cause nuclear accumulation are still unclear (M. Morgan and A.

Thorburn, unpublished data). Similarly, FADD has been reported to have nuclear import and export sequences and to be primarily localized in the nucleus [43,44]. It is not yet clear how the nuclear localization of these adaptors is related to their death receptor activities and it is possible that these unexpected localizations indicate a different function for these molecules. For example, nuclear FADD may be involved in the response to DNA damage because nuclear FADD is associated with the methyl–CpG binding domain protein 4, which is involved in the mismatch repair apparatus [43]. Similarly, apoptosis by an exclusively nuclear TRADD truncation mutant could be distinguished from caspase-8-dependent apoptosis (i.e. the kind of apoptosis that we expect to be induced by activated death receptors) [42], further suggesting a role that is distinct from death receptor signalling. Indirect evidence for signalling from intracellular compartments by components of the death receptor complexes comes from studies with LMP1, a viral protein that mimics some death receptor signalling pathways. This molecule uses many of the same signalling proteins as the death receptors but while it can be localized at the cell membrane, the signalling occurs from intracellular compartments [45].

While we cannot exclude the possibility that the nuclear and other intracellular signalling by these proteins may be important mainly in response to stimuli other than death ligands, these findings lend support to the view that there are important functions for these proteins at locations other than the cell membrane. By determining where within the cell the initial caspase activation events in response to death ligands occur, using Fluorescence Resonance Energy Transfer to measure caspase activity [46–50], it should be possible to determine how these activities fit into the death receptor signalling pathways.

5. How many ligand-bound receptors are needed to induce apoptosis?

Soluble death ligands such as Fas ligand (FasL) are not as potent as membrane-bound ligands. An explanation for this observation comes from the finding that a single FasL molecule bound to a Fas receptor trimer is not able to induce apoptosis. Interestingly, apoptosis (and other Fas-dependent signalling including JNK phosphorylation) can be restored if two soluble FasL molecules are physically linked [51]. Thus the active death receptor complex that is able to signal apoptosis probably consists of at least two ligands bound to a hexameric receptor consisting of two pre-formed trimers [18]. These data explain why soluble epitope-tagged versions of death ligands are significantly more pro-apoptotic if they are crosslinked by antibodies that recognize the tag. Why must we have two receptor trimers each bound by linked ligands in order to activate caspase-8? One simple explanation is that a single ligand-bound receptor trimer is unable to bind two procaspase-8 molecules and thus unable to promote caspase dimerization. Determination of the stoichiometry of the various components of the DISC (i.e. receptors, FADD, procaspase-8, etc.), combined with structural analysis of active and inactive DISCs, should provide an answer to this question. Differences in the number of receptors that must be activated to induce efficient death signalling may have important practical consequences for the development of therapeutics based on death receptor agonists and antagonists.

6. Caspase-independent death induced by death receptors

It was initially thought that all death receptor-induced cell killing was achieved through caspase activation and the above discussion has focused on how caspase-dependent cell death occurs. However, there are numerous reports of programmed cell death in response to activation of death receptors even when caspases are inhibited, suggesting that caspase-independent pathways can be stimulated by death receptors. Examples include death induced by Fas or TNFα that has been characterized as necrotic [52–54].

In at least some cases, the decision to undergo caspase-dependent apoptosis or caspase-independent necrosis may involve the heat shock protein HSP90, which may alter the components of the DISC to activate either apoptosis or necrosis [55]. Fas-induced necrosis has also been reported to require signalling through the DISC-interacting kinase RIP [56]. While this response (unlike some other RIP-dependent response such as NFκB signalling) requires the kinase activity, the relevant substrate is not known. There have been few studies that identify the effectors (i.e. proteases other than the caspases) that mediate death receptor-induced death when caspases are not involved. An exception is the identification of the lysosomal protease, cathepsin B, as a mediator of TNF-induced cell death in some cancer cell lines [57]. Importantly, this pathway was not activated in other tumour cell lines, indicating that different cell types may be more or less able to activate non-caspase dependent signalling even in response to the same receptor activation events. Unlike the case with caspase activation, we have little understanding of how alternate effectors like cathepsin B are activated by the receptors.

Recently, our laboratory found that TRAIL could kill normal prostate epithelial cells through pathways that involve both caspases and a serine protease that seem to work in parallel pathways [58]. Importantly, the serine protease-dependent arm was not involved in killing of prostate cancer cells. This last finding further emphasizes that while the caspase activation pathway involving FADD-dependent caspase-8 recruitment works (as far as we can tell) in the same way in many if not all cell types, the caspase-independent mechanisms that death receptors use to kill cells may be highly cell-type dependent. Moreover, as in the case of prostate epithelial cells

responding to TRAIL, there may also be important differences that depend upon disease status. It seems likely that such specificity could have important biological significance but we will need to understand how these pathways are activated and controlled before we can fully interpret these differences. Most, perhaps all, the death receptors also activate other signalling pathways, e.g. to lead to NFκB, JNK or ERK signalling. In most cases, these pathways are thought to mediate pro-survival or growth signals but under some circumstances they may also participate in death signalling. Again, this may be cell- and tissue-type specific.

7. Some important remaining questions

A simple model of death receptor-induced apoptosis involving homotypic interactions between death domains on trimerized receptors and FADD and death effector domains on FADD and caspase-8 leading to caspase-8 cleavage and activation has become widely accepted. This model seems to be broadly correct but, as outlined above, new layers of complexity and opportunities for regulation are being added to it. Several important aspects remain obscure and need to be addressed experimentally. Structural studies of full-length proteins rather than the isolated domains that have been studied to date will be necessary if we are to understand how different domains (e.g. the death domain and death effector domain of FADD) interact with each other and with their binding targets. Similarly, we are just beginning to understand the stoichiometry of the death receptor complexes that signal cell death and we are just now coming to realize that the particular arrangement (e.g. of FLIP and caspase-8 molecules) and the location within the cell where these interactions occur might do more than switch apoptotic signalling on and off but could perhaps provide more subtle regulation and the activation of different apoptotic mechanisms. It will be important to understand these aspects of death receptor signalling because they may represent important regulatory steps that could be useful for targeted interventions. An obvious gap in our knowledge concerns the mechanisms and functional significance of caspase-independent death signalling by these receptors. We know it happens and we know some of the players that may be involved in the response but we have a long way to go to understand caspase-independent death signals even at the incomplete level of our understanding of caspase signals. Fortunately, we now have many of the tools to tackle these problems, so rapid progress seems assured. No doubt this progress will involve more surprising findings.

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Selective Inactivation of a Fas-associated Death Domain Protein (FADD)-dependent Apoptosis and Autophagy Pathway in Immortal Epithelial Cells

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Although evasion of apoptosis is thought to be required for the development of cancer, it is unclear which cell death pathways are evaded. We previously identified a novel epithelial cell death pathway that works in normal cells but is inactivated in tumor cells, implying that it may be targeted during tumor development. The pathway can be activated by the Fas-associated death domain (FADD) of the adaptor protein but is distinct from the known mechanism of FADD-induced apoptosis through caspase-8. Here, we show that a physiological signal (tumor necrosis factor-related apoptosis-inducing ligand) can kill normal epithelial cells through the endogenous FADD protein by using the novel FADD death domain pathway, which activates both apoptosis and autophagy. We also show that selective resistance to this pathway occurs when primary epithelial cells are immortalized and that this occurs through a mechanism that is independent of known events (telomerase activity, and loss of function of p53, Rb, INK4a, and ARF) that are associated with immortalization. These data identify a novel cell death pathway that combines apoptosis and autophagy and that is selectively inactivated at the earliest stages of epithelial cancer development.

INTRODUCTION

Because apoptosis can suppress tumor development, it is sometimes thought that cancer cells are generally resistant to apoptosis, whereas normal cells are sensitive. In fact, cancer cells are closer to their apoptotic threshold than their normal counterparts and often die more easily than normal cells in response to apoptotic stimuli (Evan and Vousden, 2001; Lowe *et al*., 2004). Apoptosis sensitization in cancer cells occurs because growth-promoting oncogenic events such as Myc expression (Evan and Littlewood, 1998; Evan and Vousden, 2001; Pelengaris *et al*., 2002), Rb inactivation (Chau and Wang, 2003), E2F activation (Nahle *et al*., 2002), and cyclin D3 expression (Mendelsohn *et al*., 2002) raise the levels of apoptotic proteins or make it easier to activate these molecules and thus reduce the threshold at which apoptosis is activated. Activated oncogenes can also sensitize cells to apoptosis by promoting loss of inhibitors of apoptosis that exist in primary cells (Duelli and Lazebnik, 2000). Immortalization and transformation also sensitize cells to nonapoptotic death (Fehrenbacher *et al*., 2004).

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Abbreviations used: DD, death domain; FADD, Fas associated death domain protein; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.

If cancer cells die more easily than their normal counterparts, which cell death pathways are evaded during tumor development? One answer is that cancer cells must remain below the lowered apoptotic threshold for undergoing stress-induced apoptosis that is caused by the oncogenes that drive cell growth. Indeed, it has been suggested that this may be sufficient to cause cancer without any other cellular defects (Green and Evan, 2002). However, this model does not exclude the possibility that there may also be specific cell death pathways that inhibit cancer development in normal cells that are specifically inactivated during tumor development. Such a pathway would be expected to have the unusual characteristics of working in normal cells but not in cancer cells, and signaling proteins and physiological stimuli that activate this kind of pathway should kill normal cells by mechanisms that are selectively inhibited during the transformation process without affecting other cell death pathways.

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a promising treatment for cancer that kills tumor cells with little toxicity to normal tissues in preclinical models (LeBlanc and Ashkenazi, 2003). TRAIL induces apoptosis by binding to two receptors (DR4 and DR5) that contain an intracellular death domain (DD). Ligand binding is thought to result in conformational changes that expose a binding surface for the Fas-associated death domain (FADD) adaptor protein (Thomas *et al*., 2004a,b). FADD also binds to procaspase-8, resulting in caspase-8 dimerization and activation (Boatright *et al*., 2003; Boatright and Salvesen, 2003; Donepudi *et al*., 2003), eventually leading to effector caspase activation. This well-established mechanism causes caspase-dependent apoptosis, which can be blocked by caspase inhibitors or a dominant negative version of FADD (FADD-DD also known as FADD-DN) that has an intact DD

but lacks the death effector domain and cannot bind procaspase-8.

We previously identified an alternate method by which FADD, through its DD alone can kill cells (Morgan *et al*., 2001; Thorburn *et al*., 2003). FADD-DD–induced death was unexpected because this molecule is a widely used inhibitor of apoptosis and is unusual because it occurs in primary normal epithelial cells but not in tumor cell lines and involves both the activation of caspases through caspase-9 (not caspase-8) and a separate activity that can be blocked by a serine protease inhibitor [4-(2-aminoethyl)benzenesulfonyl fluoride; AEBSF] (Thorburn *et al*., 2003). Our previous studies raise several questions. Can a physiological stimulus activate the FADD-DD pathway or is it only induced by overexpression? What is the nature of the caspase-independent cell death that occurs in response to FADD-DD? And, when during epithelial cell transformation do cells lose the ability to respond to this pathway? Here, we answer these questions by showing that the FADD-DD pathway can be activated by a physiological signal (TRAIL receptor activation) working through the endogenous FADD protein and that when caspases are inhibited, the pathway does not kill by apoptosis but instead cells die by autophagy. We also identify a specific step in the transformation process (immortalization) when the pathway is selectively inactivated and show that this occurs via a mechanism that is separate from the known activities that occur during immortalization. These data identify a novel programmed cell death pathway involving apoptosis and autophagy that is selectively disrupted at the earliest stages of epithelial cell transformation.

MATERIALS AND METHODS

Cell Culture and Reagents

Isolation and culturing of normal human prostate epithelial cells from tissue samples was performed as described previously (Morgan *et al*., 2001; Thorburn *et al*., 2003). Human breast epithelial cells expressing defined transforming proteins were maintained as described previously (Elenbaas *et al*., 2001). Tumor cell lines were obtained and cultured as recommended by American Type Culture Collection (Manassas, VA). The mouse mammary epithelial cells were cultured as described previously (Medina and Kittrell, 2000). Mice were obtained from the National Cancer Institute Mouse Models of Human Cancer Repository (Frederick, MD). Mammary tissue was isolated from 6- to 8-wk-old virgin mice, minced, and treated with 400 U/ml collagenase for 1.5–2 h and Pronase (1 U/ml) for 20 min. After digestion, epithelial cells were separated in a Percoll gradient and then cultured on collagen-coated plates in supplemented DMEM/F-12 medium with 1% fetal bovine serum. Recombinant human TRAIL was obtained from Calbiochem (San Diego, CA) and used at 100 ng/ml; zVAD.fmk was obtained from Alexis (San Diego, CA) and used at 0.1 mM. 3-methyladenine (3-MA), cycloheximide, and H33258 (bis-benzimide, no. 33258; Aventis, Strasbourg, France) were obtained from Sigma-Aldrich (St. Louis, MO) and used at 10 mM, 0.8 μg/ml, and 10 μg/ml, respectively. Antibodies for Western blotting experiments were obtained from Cell Signaling Technology (Beverly, MA).

Microinjection, Adenovirus Infection, and Cell Death Assays

Single cell-based microinjection experiments and cell death/survival assays were performed as described previously (Thorburn *et al*., 2003). Fifty to 100 cells were injected for each plasmid in each experiment. Each injected cell was identified by virtue of its yellow fluorescent protein (YFP) fluorescence, and its fate was determined after incubation for 20 h. Because ~100 cells were injected at a time for each treatment, it was not possible to perform Western blotting to assess the expression level for YFP, YFP-FADD-DD, or the mutant proteins. Expression levels were therefore determined by visually assessing the amount of YFP fluorescence. The injected cell displayed similar levels of fluorescence, indicating that equivalent levels of each protein was compared. The percentage of living flat intact cells (rounded cells were scored as dead) was calculated for each experiment and the mean percentage of survival ± SD was calculated from at least four separate experiments by using different preparations of cells and plasmids. Survival >100% indicates that the cells grew during the experiment. Adenovirus purifications were performed using $CsCl_2$ centrifugation of doxycycline-regulated AdpEYFPc1, AdpEYFPc1-FADD-DD, and Tet repressor adenoviruses together with AdpEYFPc1-FADD-DD point mutant (V108E), which was constructed as described previously (Thorburn *et al*., 2003). Cells were infected with ~20 plaque-forming units/cell of each virus for 4 h at which time the virus-containing medium was replaced with regular tissue culture medium. Adenoviral gene expression was repressed with 1 μg/ml doxycycline, and expression was induced by removing doxycycline. These conditions produced >90% infection efficiency as determined by YFP fluorescence. Population-based cell viability assays after adenovirus infection and treatment with TRAIL, and protease inhibitors were performed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTS) assay from Promega (Madison, WI) as described in the manufacturer's instructions. Time-lapse microscopy was performed in an environmental chamber attached to a Zeiss Axiovert S200 microscope by using a 32× objective. Images were captured at 15-min intervals by using a Hamamatsu charge-coupled device (Malvern, PA) camera run by Openlab (Improvision, Warwick, United Kingdom) software, saved in QuickTime Movie format, and frames were captured for still images.

Autophagy Assays

For electron microscopy, cells were cultured in 6-cm dishes, treated with TRAIL or infected with FADD-DD–expressing adenoviruses in the presence of the caspase inhibitor zVAD.fmk as indicated on the figure legends and incubated overnight. Cells were fixed with 2.5% phosphate-buffered gluteraldehyde, postfixed in 1% phosphate-buffered osmium tetroxide, embedded in Spurr's resin, sectioned, double stained with uranyl acetate and lead citrate, and analyzed using a Philips 400 transmission electron microscope. For each treatment and control group, 20–50 randomly chosen cells were analyzed for morphological features associated with autophagy. Cells were scored as autophagy positive by using a scoring method described by Yu *et al*. (2004), where cells with <10 vesicles/cell were scored as normal, 10–19 vesicles/cell were scored as mild autophagy, 20–29 vesicles/cell were scored as moderate autophagy, and >30 vesicles/cell were scored as severe autophagy. The histograms show the percentage of cells in each category. The percentage of the total cell area taken up by autophagic vesicles for each randomly chosen cell was determined using Adobe Photoshop software. For analysis of green fluorescent protein (GFP)-LC3 localization, cells were injected with the expression plasmid along with FADD-DD or control expression plasmids, and time-lapse fluorescence microscopy was performed. Still images were captured from the movies.

RESULTS

A Physiological Stimulus Can Activate the FADD-DD Pathway through the Endogenous FADD Protein

We previously made the surprising discovery that the death domain of FADD can kill normal epithelial cells (Morgan *et al*., 2001) and showed that FADD-DD–induced cell death involves both caspases and an activity that can be inhibited by AEBSF (Thorburn *et al*., 2003) that cause different morphological phenotypes in the dying cells. To determine whether a physiological stimulus working through the endogenous FADD protein also could activate this pathway, we examined TRAIL receptor signaling. We reasoned that if TRAIL can activate the FADD-DD–dependent pathway, TRAIL-induced death of normal cells should be inhibited only when caspases and serine proteases are blocked simultaneously. In contrast, caspase inhibitors such as zVAD.fmk alone should block TRAIL-induced death in cancer cells.

For these experiments, we treated normal primary human prostate cells (sensitive to FADD-DD) or DU145 prostate cancer cells (insensitive to FADD-DD) with recombinant TRAIL in the presence of low doses of cycloheximide, which inhibited protein synthesis by ~70% (our unpublished data) and was unable to induce cell death by itself (Figure 1A). Cycloheximide treatment was required in both the normal and cancer cells to allow TRAIL-induced cell death. Cell death was monitored by time-lapse microscopy after treatment with zVAD.fmk and AEBSF. TRAIL killed both cell types, and in both cases the morphology of the dying cells was consistent with apoptosis. However, although zVAD-.fmk alone was able to block cell death in cancer cells, only the combination of zVAD.fmk and AEBSF could inhibit cell death in normal cells. The caspase inhibitor on its own did,

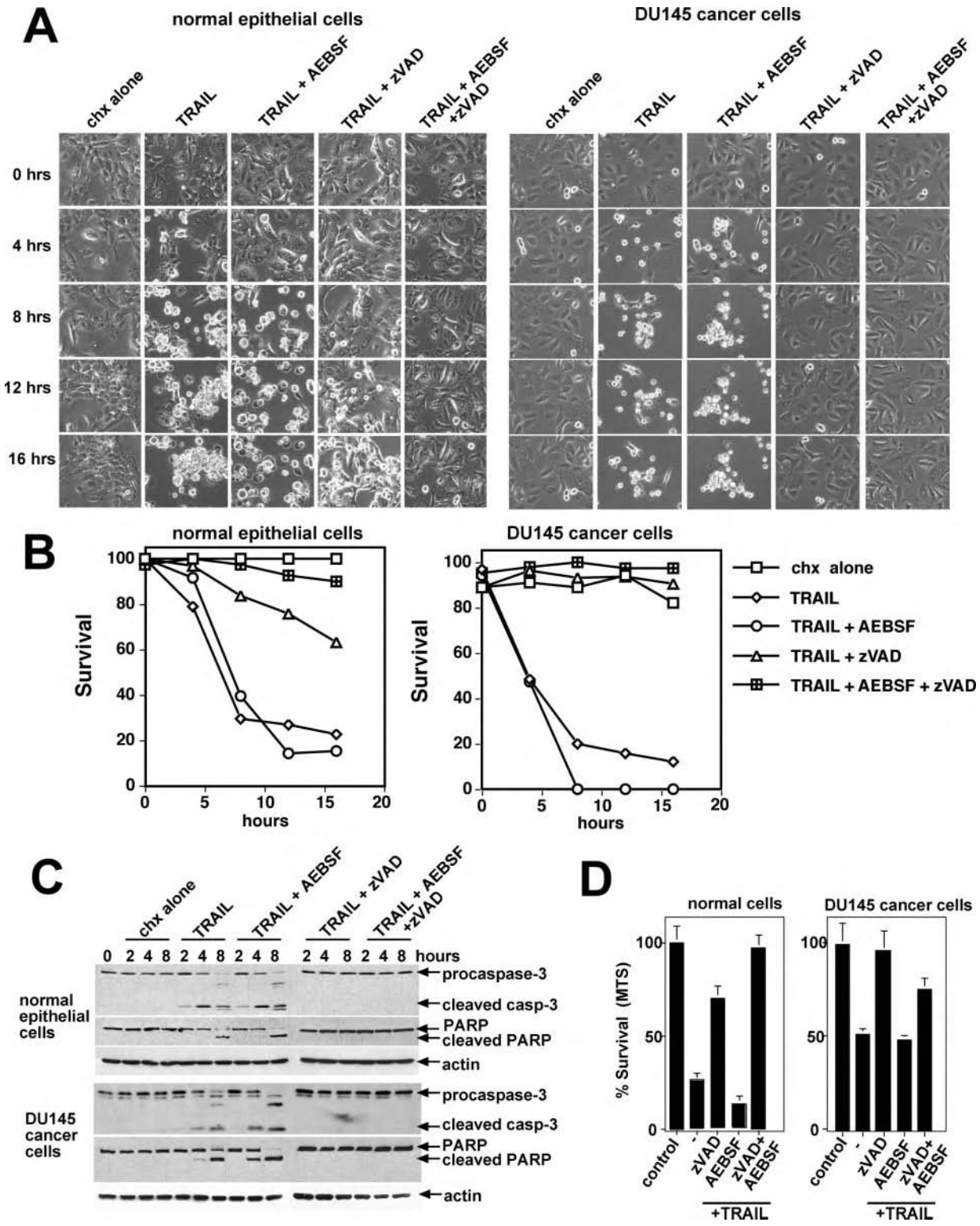

Figure 1. TRAIL can kill normal and cancerous epithelial cells by different mechanisms. (A) Time-lapse microscopy of normal prostate epithelial cells or prostate cancer cells treated with TRAIL plus AEBSF and zVAD.fmk. TRAIL kills both cell types, but zVAD.fmk alone can protect only the cancer cells; the combination of zVAD.fmk and AEBSF is required to protect normal cells from TRAIL-induced death. (B) The number of dead cells for each time point was determined by counting rounded cells in individual frames for each treatment. Treatment with zVAD.fmk in the normal cells altered the slope of the line, indicating that the caspase-independent cell death response in normal cells occurred more slowly than caspase-dependent cell death. (C) Normal or cancerous prostate cells were treated with TRAIL in the presence of the protease inhibitors and harvested for Western blot analysis of caspase-3 and PARP cleavage. In both cell types, caspase-3 was activated, leading to PARP cleavage, and the caspase inhibitor zVAD.fmk completely blocked the response. (D) MTS assays of TRAIL treated cells were performed. ZVAD.fmk only partially protected normal cells but completely protected cancer cells. The combination of zVAD.fmk and AEBSF completely protected normal cells.

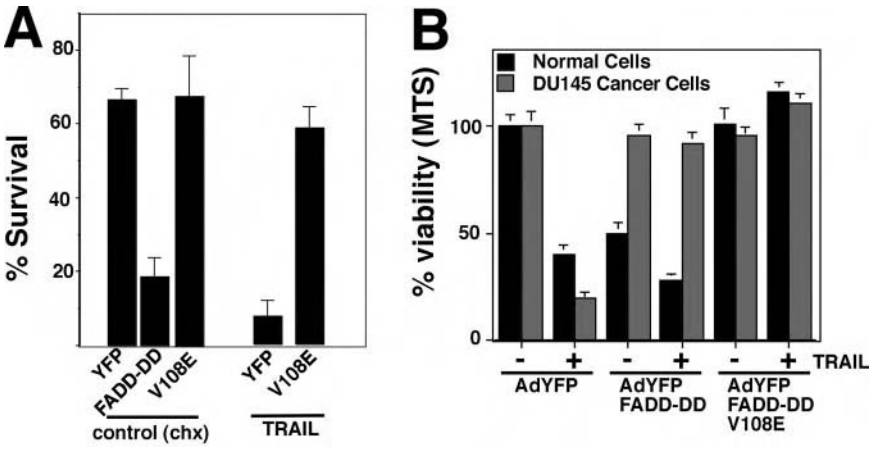

Figure 2. TRAIL activates the FADD-DD pathway through the endogenous FADD protein. (A) Normal prostate epithelial cells were injected with YFP control, FADD-DD, or FADD-DD V108E expression constructs in the presence or absence of TRAIL as indicated, and cell death was determined by monitoring the response of each injected cell. In the absence of TRAIL, FADD-DD induced apoptosis but the V108E mutant did not. The V108E mutant blocked TRAIL-induced cell death. (B) Normal cells or cancer cells were infected with doxycycline-regulated adenoviruses expressing YFP, FADD-DD, or FADD-DD V108E as indicated and then treated with or without TRAIL. Cell survival was determined using an MTS assay. TRAIL killed both normal and cancer cells, and in the absence of TRAIL, FADD-DD could kill only normal cells. In cancer cells, both the V108E mutant and the wild-type FADD-DD were equally effective at inhibiting TRAIL-induced cell death. In the normal cells, wild-type FADD-DD plus TRAIL led to increased cell death compared with either FADD-DD or TRAIL alone, whereas the V108E mutant completely inhibited TRAIL-induced cell death. These data indicate that FADD-DD functions differently in normal and cancerous prostate cells and can cooperate with TRAIL to increase normal cell death.

however, alter the morphology of the normal cells as they died in response to TRAIL. High doses of zVAD.fmk have nonspecific effects such as inhibition of cathespin B (Schotte *et al*., 1999) that have been implicated in apoptosis regulation. Therefore, our data indicate that inhibition of either caspases or such nonspecific targets is sufficient to prevent death of the normal epithelial cells. Inhibition of caspases resulted in cell death that was associated with cell rounding and detachment but little if any membrane blebbing or cellular fragmentation. Quantitation of the number of dying cells for each treatment during the time course of the experiment (Figure 1B) confirmed that zVAD.fmk alone was able to prevent cancer cell death but had only a partial effect in normal cells. Interestingly, the partial inhibition of normal cell death by the caspase inhibitor also displayed different kinetics as demonstrated by the reduced slope of the line in the time course. These data suggest that the preferred mode of death in the normal cells is via caspase-dependent apoptosis and that the cell death that occurs when caspases are inhibited is slower. This conclusion is also supported by the fact that in the absence of inhibitors the morphology of both normal and cancer cells dying in response to TRAIL is consistent with classical apoptosis with membrane blebbing, cell contraction, and fragmentation. To confirm that caspases were inhibited in both cell types by zVAD.fmk, we assessed the processing of caspase-3 and its substrate poly(ADP-ribose) polymerase (PARP) (Figure 1C). In both normal and cancer cells, the caspase inhibitor completely blocked caspase-dependent cleavage, whereas AEBSF had no effect. Similar results were obtained using MTS assays for viability in normal cells or cancer cells treated with TRAIL (Figure 1D). Together, these data indicate that under these conditions, TRAIL can kill normal cells and cancer cells by different mechanisms with normal cells displaying caspase-independent cell death in addition to caspase-dependent effects. In contrast, and in agreement with a large number of published studies in various cancer cell lines, cancer cells die by caspase-dependent apoptosis in response to TRAIL.

If TRAIL can activate the FADD-DD pathway through the endogenous FADD protein, an FADD-DD mutant that cannot cause cell death when it is expressed in normal cells should function as a dominant negative inhibitor of TRAIL-induced apoptosis. Such a molecule will also be unable to activate caspase-8 because it lacks the DED and blocks death in cancer cells, too. In contrast, the wild-type FADD-DD molecule should cooperate with TRAIL to increase normal cell death through the FADD-DD pathway but inhibit TRAIL-induced cancer cell death because this should occur only through the established caspase-8–dependent pathway. We tested several point mutants and identified a mutant (V108E) that is unable to induce normal epithelial cell death when injected into cells on its own but is able to bind to TRAIL receptors (Thomas *et al*., 2004a) and can block TRAIL-induced cell death (Figure 2A).

We expressed wild-type FADD-DD or the V108E mutant in a population of cells from a doxycycline-regulated adenovirus and then treated the normal and cancer cells with TRAIL and measured the response by using a population-based cell viability assay. Figure 2B shows that wild-type FADD-DD increased TRAIL-induced death in normal epithelial cells but blocked TRAIL-induced death in cancer cells. In contrast, the V108E mutant blocked TRAIL-induced death in both normal cells and cancer cells. These data suggest that TRAIL can work through the FADD-DD pathway in normal epithelial cells and that this pathway cannot be activated in cancer cells where all TRAIL-induced cell death occurs through the established caspase-8 pathway.

FADD-DD and TRAIL Can Induce Autophagy in Normal Epithelial Cells

Autophagy has been implicated in tumor suppression (Edinger and Thompson, 2003; Qu *et al*., 2003; Yue *et al*., 2003; Alva *et al*., 2004; Gozuacik and Kimchi, 2004) and has been linked to TRAIL-induced epithelial cell death (Mills *et al*., 2004). We therefore tested whether autophagy occurs in FADD-DD–expressing normal epithelial cells by using transmission electron microscopy (TEM). Normal epithelial cells expressing adenoviral FADD-DD had numerous membrane-bound vesicles often containing organelles and other cellular fragments (Figure 3). In contrast no significant increase in vesicles was found when the cells expressed the V108E FADD-DD mutant that is unable to kill. Similar vesicle formation occurred in normal cells after treatment with TRAIL. Vesicle formation, which is characteristic of autophagy (Gozuacik and Kimchi, 2004), occurred in the presence of the caspase inhibitor zVAD.fmk, indicating that it is separate from the caspase-dependent apoptosis that occurs in

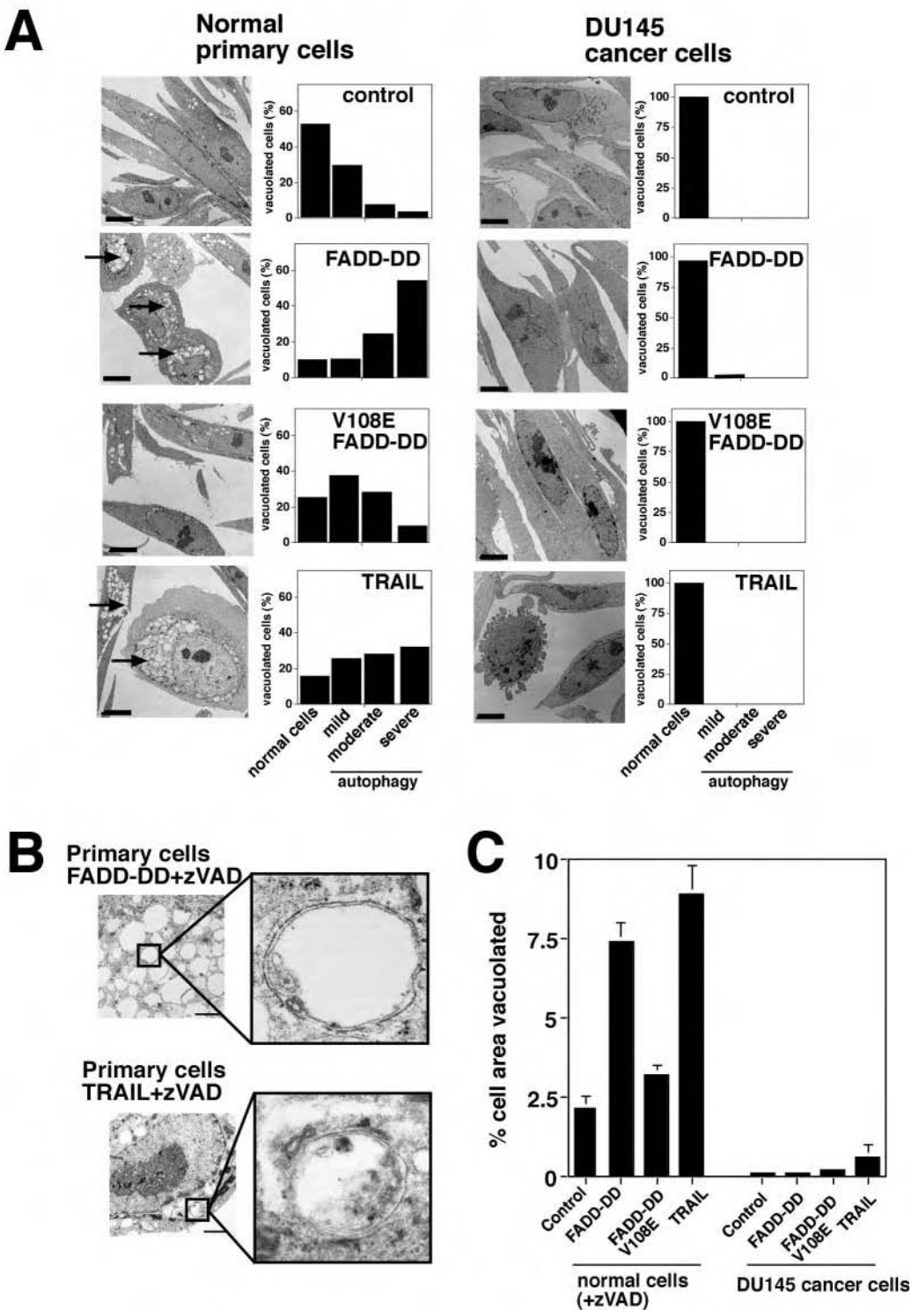

Figure 3. FADD-DD can cause autophagic vesicle formation in normal epithelial cells. (A) Normal primary prostate epithelial cells or DU145 prostate cancer cells were treated with TRAIL or infected with adenoviruses expressing FADD-DD or V108E FADD-DD as indicated and analyzed by TEM. Large numbers of vesicular structures (arrows) were found in normal cells expressing FADD-DD or treated with TRAIL. Normal cells were treated with zVAD.fmk to prevent caspase-dependent signaling from obscuring any caspase-independent effects. Bars, 5 μm. (B), higher power images of autophagic vesicles from FADD-DD or TRAIL-treated normal prostate cells showing double membranes and cellular debris. Bar, 0.5 μm. (C) Cell area taken up by autophagic vesicles, indicating that FADD-DD and TRAIL increase the proportion of each normal cell that is vacuolated.

the normal cells expressing FADD-DD or treated with TRAIL and implying that it participates in the caspase-independent arm of the cell death pathway that is induced by FADD-DD. Consistent with this idea, vesicles were not formed in response to FADD-DD or TRAIL in prostate cancer cells.

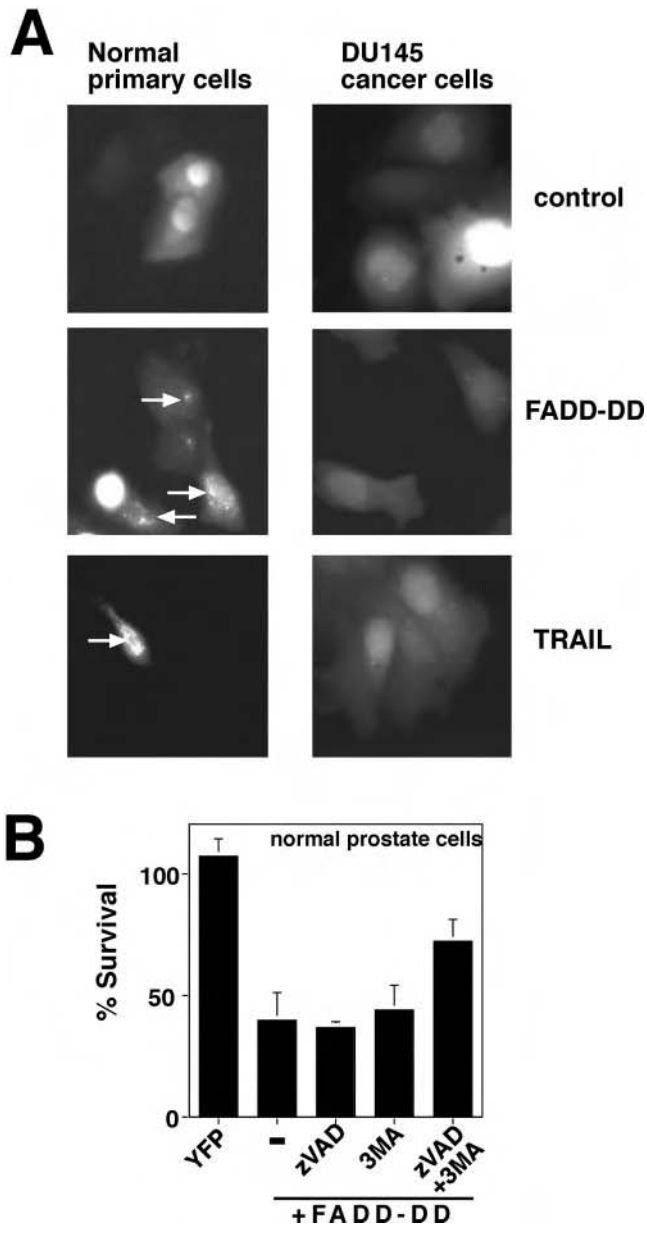

Figure 4. FADD-DD-induced autophagy in normal cells. (A) Normal prostate cells or DU145 cancer cells were injected with GFP-tagged LC3 plus FADD-DD or treated with TRAIL and followed by fluorescence microscopy. GFP-LC3 forms aggregates (arrows) in FADD-DD–expressing or TRAIL-treated normal cells but does not aggregate in cancer cells. (B) Normal prostate cell survival 24 h after injection with FADD-DD and treatment with zVAD.fmk or 3-MA alone or in combination. FADD-DD–induced cell death is not prevented by either inhibitor alone but is inhibited by the combined inhibitors.

Another characteristic of autophagy is the translocation of LC3 to autophagic vesicles, which can be detected as aggregates of GFP-tagged LC3 (Kabeya *et al*., 2000). We therefore injected normal primary epithelial cells or cancer cells with untagged FADD-DD or V108E expression vectors along with a GFP-tagged LC3 protein. The aggregation of GFP-LC3 into dots was assessed by fluorescence microscopy (Figure 4A). Aggregation of LC3 occurred in response to FADD-DD in normal cells but not in cancer cells, aggregation started before any morphological signs of cell death were apparent, and aggregation was not affected by the caspase inhibitor zVAD.fmk (our unpublished data). TRAIL treatment of the cells in the presence of zVAD.fmk had a similar effect. To test whether autophagy contributes to the FADD-DD and TRAIL-induced death that occurs in normal cells, we asked whether the autophagy inhibitor 3-MA could block cell death either on its own or in combination with zVAD.fmk. 3-MA was unable to prevent FADD-DD–induced cell death on its own but did prevent cell death when combined with zVAD.fmk (Figure 4B). These data indicate that autophagy is involved in the caspase-independent cell death response to the FADD-DD signaling pathway in normal epithelial cells.

Selective Disruption of FADD Death Domain-induced Cell Death Occurs When Epithelial Cells Are Immortalized

A distinctive feature of the FADD-DD cell death pathway is that it works in normal prostate epithelial cells but does not work in cancer cells. This raises the question of whether other epithelial cell types behave similarly and, more importantly, when during the transformation process resistance to this pathway arises. To address these questions, we examined human breast epithelial cells that were immortalized and transformed by defined genetic changes (expression of the telomerase catalytic subunit (TERT), SV40 Large T and small t antigens, and oncogenic Ras) (Elenbaas *et al*., 2001). The cells were derived by expressing the transforming proteins in normal primary human mammary epithelial cells (HMECs) and thus represent a set of cells at different steps in the transformation process arising through defined genetic changes. FADD-DD was expressed in each set of cells by microinjection, and cell death was determined by following the fate of each FADD-DD–expressing cell. Figure 5A shows that normal HMECs and the TERT-expressing HME cells were sensitive to FADD-DD–induced cell death; however, HMECs expressing TERT plus SV40 Large T antigen (HMLcE), Large T and small t antigens (HML), TERT, Large T and small t (HMLE), and cells expressing TERT, Large T, small t, and active Ras (HMLPR) were all resistant to FADD-DD–induced death.

We next asked whether this resistance to cell death was specific to the FADD-DD–induced pathway by comparing the ability of FADD-DD, which cannot activate the caspase-8 pathway, and a full-length FADD protein that can bind caspase-8, to kill HME and HMLcE cells. A general apoptosis resistance mechanism arising in the immortal HMLcE cells should inhibit both FADD proteins. In contrast, a mechanism that selectively disrupts the FADD-DD pathway in HMLcE cells should not alter cell death in response to the FADD molecule that can activate caspase-8. HMLcE cells were resistant to FADD-DD, whereas both HME and HMLcE cells were killed equally well by full-length FADD (Figure 5B). These data indicate that selective resistance to FADD-DD–induced killing arises at a specific step during transformation and can be conferred by a viral oncogene (SV40 Large T antigen). These data also show that the FADD-DD pathway is not affected by TERT expression.

The TERT and T antigen-expressing HMECs are immortal but not transformed (Elenbaas *et al*., 2001), suggesting that resistance to FADD-DD–induced cell death is associated with immortalization rather than transformation. We therefore tested whether spontaneously immortalized epithelial cells are resistant to FADD-DD. Because human cells very rarely undergo spontaneous immortalization, we used mouse epithelial cells and compared the response to FADD-DD in primary low passage cells to cells that had undergone spontaneous immortalization after continued culture. We also compared the re-

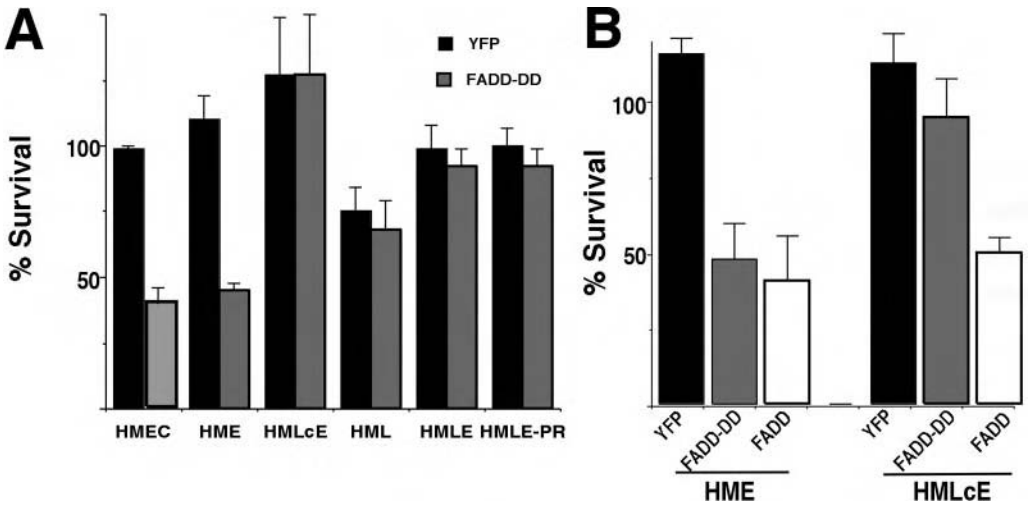

Figure 5. FADD-DD–induced cell death is selectively inhibited in immortalized cells. (A) HMECs at different stages of immortalization and transformation were injected with YFP control or YFP-FADD-DD expression vectors, and the percentage of survival for fluorescent cells was determined. FADD-DD killed normal HMECs and TERT-expressing HME cells, but it did not kill HMEC expressing T antigen plus TERT (HMLcE) or any of the other cells. Panel B, HME cells and HMLcE cells were injected with control, FADD-DD, or a full-length FADD construct that can activate caspase-8. Both FADD-DD and FADD could kill HME cells, but only the FADD molecule capable of activating caspase-8 killed HMLcE cells. These data show that resistance to FADD-DD–induced cell death arises in response to expression of T antigen, which causes immortalization and that this resistance is specific to the FADD-DD pathway.

sponse in primary fibroblasts from the same tissue pieces to test whether the response was epithelial specific. Figure 6A shows that primary mouse mammary epithelial cells (MMECs) were killed by FADD-DD, whereas spontaneously immortalized epithelial cells and primary nonimmortalized fibroblasts were resistant. All the cells underwent apoptosis in response to the full-length FADD protein that can activate caspase-8, indicating that the immortalized cells acquire selective resistance to the FADD-DD pathway rather than a general resistance to all apoptotic stimuli. Similar results were obtained in mouse prostate epithelial cells (our unpublished data). Together with our previous studies (Morgan *et al*., 2001; Thorburn *et al*., 2003), these data indicate that human and mouse prostate and breast epithelial cells respond to FADD-DD in the same way.

There are differences in the requirements for immortalization between cell types and between mouse and human cells (Romanov *et al*., 2001; Drayton and Peters, 2002; Rangarajan and Weinberg, 2003). However, in all cells, it is thought that disruption of p53, INK4a/ARF (these two gene products from the same locus regulate the Rb and p53 pathways), and Rb are important steps in the immortalization process (Drayton and Peters, 2002; Hahn and Weinberg, 2002; Rangarajan and Weinberg, 2003). TAg inactivates p53 and Rb (Ali and DeCaprio, 2001). Disruption of the p53 pathway might therefore provide a simple explanation for the inability of FADD-DD to kill immortal tumor cells. We therefore examined the response to FADD-DD and a FADD molecule that can activate caspase-8 in MMECs from p53 knockout animals. Figure 6B shows that low passage primary epithelial cells from the p53 knockout animals were killed in response to FADD-DD, indicating that loss of p53 function does not affect the FADD-DD pathway and excluding this explanation for the immortalization-dependent resistance. As expected the p53−/− cells did not become senescent and grew well in culture. However, upon continued culture, the cells became resistant to FADD-DD–induced apoptosis but were equally sensitive to apoptosis induced by a FADD molecule that can bind and activate caspase-8. We next cultured epithelial cells from mice with knockouts of the p53 target gene p21, which controls cell cycle progression, and INK4a/ARF (both genes are inactivated in these animals, which have a deletion of exons 2 and 3; Serrano *et al*., 1996). In each case, primary MMECs underwent FADD-DD–dependent apoptosis (Figure 6C). As with the p53 −/− cells, MMECs that lack functional INK4A/ARF genes became resistant to FADD-DD–induced apoptosis when they were continuously cultured (Figure 5D), suggesting that acquisition of selective resistance to this pathway confers an advantage to the cells.

SV40 T antigen also inactivates Rb, and we next asked whether this was responsible for resistance to FADD-DD. Because Rb knockout results in embryonic lethality (Jacks *et al*., 1992), we isolated MMECs from animals with homozygous "floxed" Rb genes. These cells were infected with an adenovirus that expresses Cre recombinase to knockout the Rb gene. Three days after infection, there was no detectable Rb protein in the cells (Figure 6C, inset). FADD-DD injection into Rb-deficient cells resulted in apoptosis induction that was equally efficient as that observed with the FADD molecule that can activate caspase-8 (Figure 6C). Together, these data indicate that the FADD-DD pathway is selectively disrupted upon immortalization but that resistance does not arise as a result of the inactivation of p53, INK4a, ARF, p21, or Rb that occurs during immortalization.

Autophagy Is Inactivated in FADD-DD-resistant Epithelial Cells

If autophagy is involved in the FADD-DD pathway, it should occur in early passage mouse breast cells that express FADD-DD and should not be inhibited by zVAD.fmk. However, when cells acquire resistance to FADD-DD–induced cell death, they should also fail to show signs of autophagy. We therefore assessed autophagic vesicle formation in response to FADD-DD in low (passage 4, i.e., sensitive to FADD-DD–induced cell death) and high (passage 30, i.e., insensitive to FADD-DD–induced death) passage MMECs from INK4a/ARF knockout animals. These cells were chosen because they come from the same primary cell prepara-

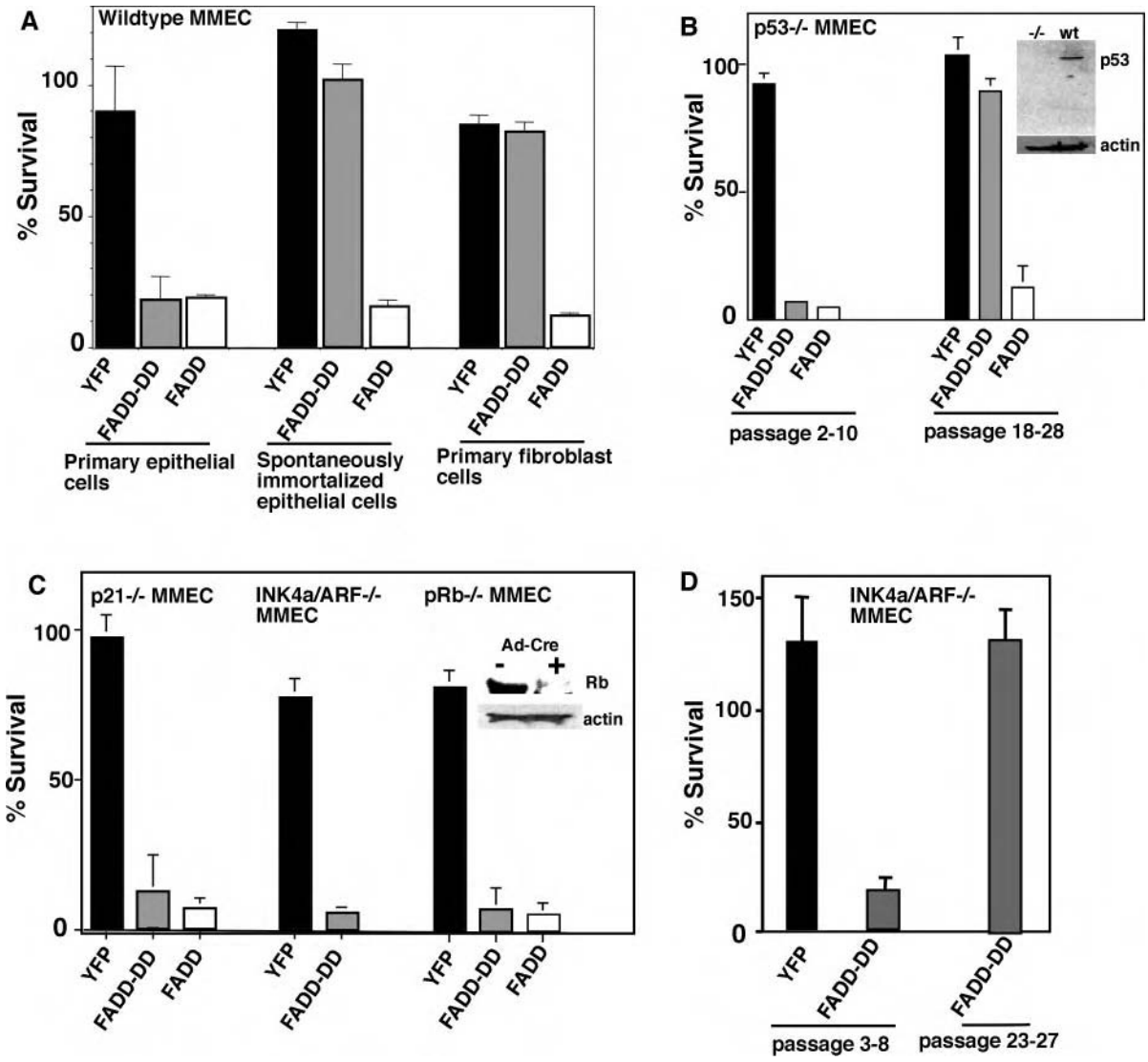

Figure 6. Inhibition of the FADD-DD pathway in immortal cells is not caused by inactivation of genes that are known to regulate immortalization. (A) Primary mouse mammary epithelial cells, spontaneously immortalized epithelial cells, or primary breast fibroblasts were injected with YFP control, FADD-DD, or FADD expression constructs, and cell survival was determined. All the cell types were killed by the FADD molecule that can activate caspase-8, but only the primary epithelial cells were killed by FADD-DD. (B) Mammary epithelial cells were isolated from p53 knockout mice and tested for sensitivity to FADD-DD after limited culture (passage 2–10) or extended in vitro culture (passage 18–28). FADD-DD killed the low passage number cells but could not kill the high passage number cells. The Western blot insert compares protein samples from the p53 knockout or wild-type animals showing that the cells lacked p53. (C) Primary MMECs were cultured from p21 and INK4a/ARF knockout animals or from animals with floxed Rb genes, which were subsequently infected with a Cre recombinase adenovirus and maintained for 3 d in culture at which time no detectable Rb protein was present (inset). All the low passage (< passage 10) primary cells underwent apoptosis in response to FADD-DD. (D) Low passage (passage 3–8) or high passage (passage 23–27) MMECs from INK4a/ARF −/− mice were injected with FADD-DD as indicated. Only the low passage cells were killed by FADD-DD.

tion and both grow well in culture yet they differ in their ability to die in response to FADD-DD expression. In addition, because the cells lack INK4a and ARF, both the Rb and p53 pathways are inactivated, thus removing potential confounding influences of other cell death pathways. The cells were infected with the FADD-DD- or V108E FADD-DD–expressing adenoviruses, treated with zVAD.fmk to block caspase activation, and assessed by TEM. Low passage cells expressing FADD-DD displayed high levels of autophagic vesicle formation; however, vesicles were not formed in low passage cells expressing the V108E mutant or in high passage cells expressing either wild-type or mutant FADD-DD (Figure 7).

DISCUSSION

In this article, we present data showing that the death domain of FADD can activate a cell death pathway involving both apoptosis and autophagy that is selectively inactivated when normal epithelial cells are immortalized. The same pathway can be activated by TRAIL receptor stimulation and blocked by a FADD-DD mutant, indicating that although the pathway is conveniently activated by expression of exogenous FADD-DD, a physiological signal also can activate this pathway through the endogenous FADD protein. Prostate and breast epithelial cells behave similarly but fibroblasts are unable to activate this pathway. Resistance to

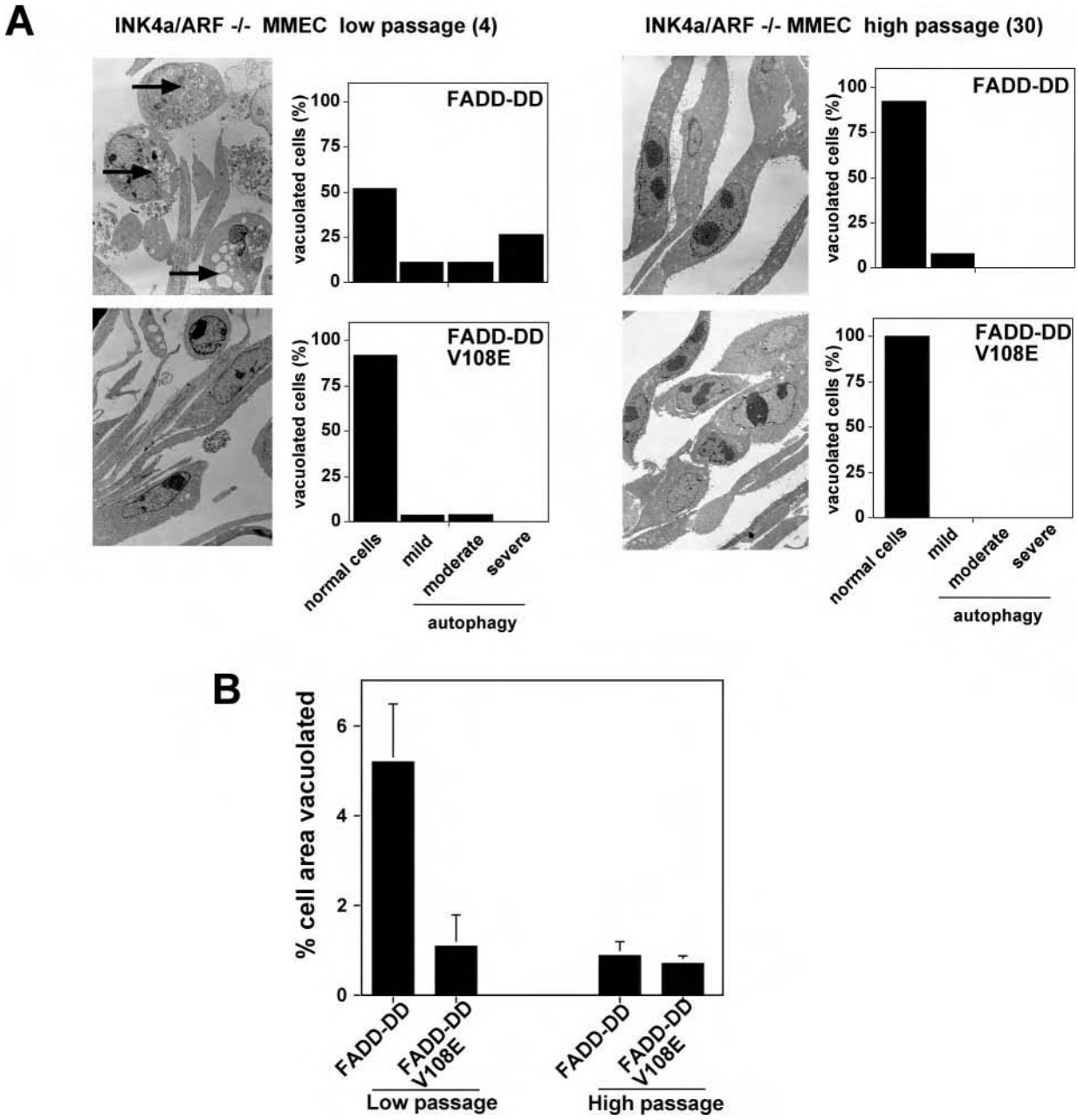

Figure 7. FADD-DD-induced autophagy is lost in late passage epithelial cells. (A) Low or high passage MMECs from INK4a/ARF knockout animals were infected with FADD-DD or V108E FADD-DD adenoviruses in the presence of zVAD.fmk to inhibit caspase-dependent effects and analyzed by TEM for signs of autophagy. Large numbers of vesicles (arrows) were observed only in the low passage cells expressing wild-type FADD-DD. (B) Cell area taken up by vacuolated structures, indicating that FADD-DD but not the V108E mutant causes an increase in such structures in low passage cells.

this form of cell death occurs without affecting other apoptosis pathways, including those that are induced by an FADD protein that can interact with and activate caspase-8 through a different part of the protein. This resistance arises at immortalization rather than complete transformation, suggesting that it represents an early cell death defect that occurs during the development of epithelial cancers. This work provides the first example that we are aware of where apoptosis and autophagy are induced in a cell type-specific manner and selectively disrupted during immortalization and transformation.

Our experiments demonstrating inhibition of TRAIL-induced death by the V108E mutant and cooperation between TRAIL and wild-type FADD-DD to increase killing of normal cells suggest that a stimulus that works through FADD can activate the FADD-DD apoptosis/autophagy pathway. It is important to note that this does not necessarily mean that the normal physiological stimulus is actually TRAIL or that the FADD-DD pathway is an important aspect of TRAIL signaling under normal circumstances. Moreover, because activation of the FADD-DD pathway by TRAIL in normal cells was only detected when we blocked the canonical

caspase-8–dependent pathway, the FADD-DD pathway may not be the major TRAIL-induced cell death pathway even if TRAIL is the physiological stimulus. Instead, it is possible that a different stimulus (perhaps not even involving death receptors) activates FADD to induce the FADD-DD pathway under normal circumstances. Because we have identified a point mutant (V108E) that cannot activate the FADD-DD pathway, it may be feasible to address these issues by creating a knockin mouse that contains the V108E mutation (which, if our ideas are correct, may have a cancer-related phenotype) and asking whether TRAIL signaling occurs properly in these animals.

We have not detected FADD cleavage in normal cells after treatment with TRAIL. In addition, we previously found that overexpression of a full-length FADD molecule containing a point mutation in the DED that prevents caspase-8 binding or expression of wild-type FADD in the presence of a caspase-8 inhibitor could kill normal but not cancerous cells (Thorburn *et al.*, 2003). We therefore do not suggest that the isolated FADD-DD protein occurs under physiological conditions or that the FADD-DD pathway is activated only by the truncated protein. Instead, we think that the FADD-DD pathway is activated by full-length FADD but that this is only evident when the canonical caspase-8 pathway is blocked. We therefore view the expression of the truncated FADD-DD protein, which provides the most effective way to activate this pathway without activating the canonical caspase-8 pathway, as a useful tool to selectively activate and study the pathway that is normally activated by the endogenous full-length FADD protein.

There are other recent examples where autophagy and apoptosis is combined. TRAIL-induced autophagy occurs during breast epithelial cell death to form acini in three-dimensional cultures (Mills *et al.*, 2004). However, this cell death, which occurred in immortal MCF10A cells, was blocked by FADD-DD, suggesting that it has some differences from the FADD-DD–induced death in nonimmortalized cells. In addition, DAP kinase, which has been implicated in death receptor-induced cell death (Cohen *et al.*, 1999), can cause autophagy in addition to apoptosis (Inbal *et al.*, 2002). Beclin 1, which promotes autophagy, is a haploinsufficient tumor suppressor (Qu *et al.*, 2003; Yue *et al.*, 2003) that displays reduced expression in breast tumors (Liang *et al.*, 1999), providing a genetic link between defects in autophagy and cancer development. Our work suggests that at least some such defects arise at the earliest steps in epithelial cancer development (i.e., the acquisition of immortalization) to inactivate specific cell death pathways that involve both caspase-dependent apoptosis and autophagy.

Although there are differences in the requirements for immortalization and transformation of human and mouse cells (Drayton and Peters, 2002; Rangarajan and Weinberg, 2003), mammary epithelial cells from both organisms behave identically in regards to FADD-DD–induced apoptosis/autophagy and are inhibited by immortalization in both cases. Prostate epithelial cells also behave the same way. Although immortalization is associated with acquired resistance to this cell death pathway, the known activities that are involved in mammalian cell immortalization, including telomerase activation, or loss of function of p53, INK4a, ARF, and pRb are not responsible for resistance to this cell death pathway. In addition, MMECs lacking p53, or INK4a and ARF, which do not undergo crisis or become senescent, become selectively resistant to the FADD-DD pathway upon continued culture. These data suggest that the acquisition of resistance to FADD-DD–induced cell death represents an uncharacterized aspect of immortalization that confers a selective advantage to the cells.

Although evasion of apoptosis is widely regarded as a hallmark of cancer (Hanahan and Weinberg, 2000), the cell death pathways that must be avoided are poorly understood. Because growth-promoting oncogenic events such as Myc expression or Rb inactivation sensitize cells to diverse apoptotic stimuli and function as an intrinsic tumor suppression mechanism (Lowe *et al.*, 2004), cancer cells must overcome this hurdle to remain below their apoptotic threshold. This can be achieved by altering components of the cell death machinery such as p53, ARF, or Bcl-2 family members that control diverse apoptotic pathways (Lowe *et al.*, 2004). The apoptosis/autophagy pathway that is induced by FADD-DD and TRAIL has unusual characteristics (normal epithelial cell specificity, inactivation when cells are immortalized without affecting other cell death pathways, and no inhibition by loss of p53 or ARF or Bcl-2 expression) that are unlike oncogenic sensitization to apoptosis and suggest it represents a specific hurdle that some cells must also overcome if they are to become cancerous. Further understanding of how the FADD-DD pathway works and why it is not able to work in immortal cells should provide new insights into the role of apoptosis and autophagy dysfunction in the development of epithelial cancers.

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