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Non-canonical β -catenin degradation mediates reactive oxygen species-induced epidermal cell death

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Abstract

β -catenin is constantly degraded through the ubiquitin-proteasomal pathway. We here report that a different type of β -catenin degradation is causally involved in epidermal cell death. We observed that reactive oxygen species (ROS) caused β -catenin degradation in the epidermal cells through a caspase-dependent mechanism, which results in disruption of cell adhesion. Disruption of cell adhesion increased ROS and activated caspases. Upregulation of the intact β -catenin blocked ROS accumulation and caspase activation. These results indicate that a feed-forward loop consisting of ROS, caspases activation and β -catenin degradation induces epidermal cell death.

Keywords

apoptosis; β -catenin; caspase; epidermis; reactive oxygen species

Introduction

β -catenin is a component of cell adherens junction (AJ) and also functions as a transcription factor involved in cell proliferation and differentiation (Clevers, 2006; Perez-Moreno & Fuchs, 2006). At steady state, cytosolic β -catenin is rapidly degraded by a destruction complex involving APC, axin, glycogen synthase kinase 3 β (GSK3 β) and casein kinase (Brembeck et al, 2006; Clevers, 2006; Xu & Kimelman, 2007). β -catenin is phosphorylated by GSK3 β , which targets it for proteasome degradation. β -catenin is an abundantly produced protein, however, through this homeostatic β -catenin degradation pathway, the level of β -catenin is kept constant. It has been reported that β -catenin can be cleaved by protease including caspases and calpains (Abe & Takeichi, 2007; Brancolini et al, 1997; Brancolini et al, 1998; Herren et al, 1998; Li & Iyengar, 2002; Steinhilber et al, 2000; Van de Craen et al,

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Conflict of Interest

The authors declare no conflict of interest.

1999). In neuronal cells, the calpain-dependent cleavage stabilizes and stimulates transcriptional activity of β -catenin (Abe & Takeichi, 2007). In other cell types, β -catenin cleavage has been reported as one of the consequences of apoptosis (Bannerman et al, 1998; Brancolini et al, 1997; Brancolini et al, 1998).

TAK1 is a member of mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK), and is an indispensable signaling intermediate in MAPK-AP-1 and NF- κ B pathways (Ninomiya-Tsuji et al, 1999; Shim et al, 2005; Takaesu et al, 2001). AP-1 and NF- κ B are major transcriptional factors of cellular antioxidant genes such as ferritin and glutamylcysteine ligase (GCL) (Go et al, 2004; Pham et al, 2004; Yang et al, 2005). Ablation of TAK1 impairs MAPK and NF- κ B pathways resulting in reduced cellular antioxidant capacity (Omori et al, 2008). We have previously reported that *tak1*-deficient cells are hypersensitive to ROS inducers because ROS are accumulated much higher levels in *tak1*-deficient cells compared to wild type cells (Omori et al, 2008). In epidermal cells (keratinocytes), we have previously found that TNF is a strong inducer of ROS in *tak1*-deficient conditions (Omori et al, 2008). In *tak1*-deficient epidermis of the skin, keratinocytes undergo TNF-ROS-induced apoptosis (Omori et al, 2008). However, the underlying mechanism through which ROS kill keratinocytes has not been fully defined. In the present study, we investigate how ROS cause keratinocyte apoptosis both in cultured cells and in an in vivo setting by using TNF-induced ROS in *tak1*-deficient cells as model systems. We observed that ROS-induced apoptosis is accompanied by caspase-dependent degradation of β -catenin. Furthermore, we found that this non-canonical β -catenin degradation is not only the result of caspase activation but is also the cause of apoptosis.

Results and Discussion

ROS cause β -catenin degradation in the in vivo epidermis

We analyzed two mouse models; one having an inducible epidermal-specific deletion of *tak1* (*K14-CreERT tak1^{flox/flox}*) and the other having a constitutive epidermal-specific deletion of *tak1* (*K5-Cre tak1^{flox/flox}*) mice (Fig. 1). TNF is always expressed at some levels in the normal skin, which is important for basal immunity (Pasparakis et al, 1996; Pasparakis et al, 1997). In the *tak1*-deficient epidermis, this TNF sufficiently induces ROS-dependent cell death (Omori et al, 2008). We treated 5-weeks old *K14-CreERT tak1^{flox/flox}* mice with 4-hydroxy tamoxifen (4-OHT) (Fig. 1A) which induced *tak1* gene deletion and increased apoptotic epidermal cells as previously reported (Omori et al, 2008). We found that β -catenin was greatly decreased in the epidermis of 4-OHT-treated *K14-CreERT tak1^{flox/flox}* mice (Fig. 1A). The amount of intact β -catenin was diminished and several smaller degraded forms of β -catenin were observed. The degradation of β -catenin was blocked by feeding the mice a diet containing antioxidant butylated hydroxyanisole (BHA), suggesting that the degradation of β -catenin is associated with increased ROS (Fig. 1A). Neonatal mice with constitutive epidermal-specific deletion of *tak1* (*K5-Cre tak1^{flox/flox}*) displayed severe dysregulation of epidermal homeostasis ((Omori et al, 2006) and Fig. 1B, 2nd panels from left). Apoptosis was highly induced in *tak1*-deficient neonatal epidermis ((Omori et al, 2006) Fig. 1B, most right panels) which was correlated with diminished membrane-bound β -catenin staining in postnatal day 4 and 5 (P4 and P5) (Fig. 1B middle

panels and Fig. 1C). Impaired membrane localization of β -catenin was observed in the most areas of P4 TAK1 mutant skin, while β -catenin was largely diminished in some areas of P5 in TAK1 mutant skin.

ROS induce β -catenin degradation and disruption of cell adhesion

To further analyze the degradation of β -catenin, we utilized cultured *tak1*-deficient (/) keratinocytes (Omori et al, 2006). We have previously reported that TNF induce accumulation of ROS and caspase activation leading to apoptotic cell death in *tak1*-deficient keratinocytes, whereas no wild type keratinocyte undergo apoptosis upon TNF stimulation (Omori et al, 2008). We found that the level of β -catenin was diminished in TNF-stimulated *tak1*-deficient (/) but not in *tak1* wild-type (+/+) keratinocytes (Fig. 2A), and restoration of wild type *tak1* (/ +TAK1) blocked the β -catenin degradation (supplementary Fig. S1A). Decreased β -catenin in TNF-stimulated *tak1*-deficient keratinocytes was correlated with accumulation of ROS and cell death (Fig. 2B, and D). The reduction of β -catenin and cell death were abolished by pretreatment of BHA (Fig. 2C and D), which is consistent with the in vivo results in mouse epidermis (Fig. 1A).

We observed cells having diffuse non-membrane-bound staining of β -catenin in TNF-treated *tak1*-deficient (/) (Fig. 2E, asterisks). 98% of diffuse β -catenin cells were co-stained with cleaved caspase 3, indicating that they were apoptotic. Furthermore, phalloidin staining displayed a punctate pattern and retraction of fibers, indicating that disruption of actin filament and cell adhesions (AJs) were associated with the loss of membrane-bound β -catenin (Fig. 2E, arrows). All the cells showing the disruption of actin filament were co-stained with cleaved caspase 3. These results suggest that β -catenin degradation is associated with disruption of AJs. We also observed decreased amounts of other AJ components, E-cadherin and p120 catenin in TNF-stimulated *tak1*-deficient keratinocytes, and the reduction of those proteins were blocked by the antioxidant BHA treatment (supplementary Fig. S1B). These results suggest that AJs were disrupted via ROS in TNF-treated *tak1*-deficient keratinocytes.

Caspases degrade β -catenin

We next examined whether the reduction of β -catenin is mediated through the GSK3 β -mediated proteasomal degradation pathway. We pretreated *tak1*-deficient keratinocytes with several types of inhibitors including proteasomal inhibitors (MG132 and lactacystin) and a GSK3 β inhibitor (LiCl), and then stimulated the cells with TNF. Degradation of β -catenin was inhibited by pretreatment of a proteasomal inhibitor, MG132 or lactacystin (Fig. 3), but inhibition of GSK3 β had almost no effect on TNF-induced β -catenin degradation. We found that a caspase inhibitor, Z-VAD-fmk (Z-VAD) abolished degradation of β -catenin and a calpain inhibitor N-acetyl-Leu-Leu-methional (ALLM) less effectively inhibited degradation of β -catenin (Fig. 3 and supplementary Fig. S1C). Therefore, it is likely that TNF-induced ROS do not degrade β -catenin through the homeostatic GSK3 β pathway, but it may be through a non-canonical pathway which involves caspases.

β -catenin degradation is causally involved in apoptosis

Epidermis is characterized by tightly connected cells (Perez-Moreno & Fuchs, 2006). AJs are important for maintenance of this tight connection, and disruption of cell-cell connection is known to be associated with apoptosis (Perez-Moreno & Fuchs, 2006; Steinhilber et al, 2000). Therefore, we postulated that β -catenin degradation-associated AJ disruption might not only be the result of caspase activation but also be causally involved in apoptosis in keratinocytes. To test this hypothesis, we attempted to prevent the reduction of the level of β -catenin, and examined TNF-induced apoptosis. We stably introduced a vector expressing β -catenin into *tak1*-deficient keratinocytes by using the retrovirus system. However, we noted that the level of β -catenin was not noticeably increased by the β -catenin vector infection in unstimulated keratinocytes. We postulated that β -catenin degradation through the GSK3 β -proteasomal pathway might mask the increased β -catenin expression. To verify this, we used S33Y- β -catenin, which is not phosphorylated by GSK3 β or degraded through the canonical pathway (supplementary Fig. S2). The expression level of S33Y- β -catenin was higher than that in the wild type β -catenin or control vector infected unstimulated keratinocytes (supplementary Fig. S2B), which is consistent with our hypothesis. We found that the stable introduction of even the wild type β -catenin vector could maintain intact β -catenin at nearly control levels following TNF stimulation (Fig. 4B and supplementary Fig. S2B). Exogenous expression of either wild type or S33Y- β -catenin reduced the number of TNF-induced morphologically apoptotic (shrink) cells and annexin V-binding positive cells (Fig. 4A and supplementary Fig. S2A). Activation of caspase 3 was found to be blocked by β -catenin overexpression (Fig. 4B). Furthermore, β -catenin overexpression could effectively block ROS accumulation (Fig. 4C).

We showed above that a proteasomal inhibitor MG132 reduced β -catenin degradation (Fig. 3). Therefore, we also examined whether MG132-mediated upregulation of β -catenin could modulate caspase, ROS and cell survival (supplementary Fig. S3). MG132 completely abolished TNF-induced caspase 3 activation and ROS accumulation. Collectively, maintenance of intact β -catenin level can block caspase 3 activation and ROS accumulation. These results indicate that degradation of β -catenin is not a consequence of apoptosis but causes ROS accumulation and apoptosis.

These results led us to hypothesize that ROS, caspase activation and β -catenin degradation form a feed-forward loop leading to keratinocyte apoptosis (see Fig. 7). If our hypothesis is correct, disrupting any of them should block all other events. Indeed, we showed above that reduction of ROS by the antioxidant BHA blocked caspase activation and β -catenin degradation (Fig. 1 and 2), and that inhibition of caspases blocked β -catenin degradation (Fig. 3). We next examined whether inhibition of caspases could block ROS accumulation. We treated *tak1*-deficient keratinocytes with Z-VAD and determined the levels of TNF-induced ROS (Fig. 5A). Inhibition of caspases completely abolished the accumulation of ROS (Fig. 5A) and reduced the number of shrinking cells and annexin V-binding positive cells (Fig. 5B) in *tak1*-deficient keratinocytes following TNF stimulation. These results are consistent with our killing loop hypothesis.

The ROS-caspase- β -catenin degradation loop kills epidermal cells

If there is such a feed-forward loop shown in Fig. 7, any of the events, including ROS accumulation, caspase activation and β -catenin degradation, should be capable of initiating the loop leading to keratinocyte death. We attempted to trigger the ROS-caspase- β -catenin degradation loop by stimuli other than the TNF-induced ROS. We mimicked β -catenin-induced AJ disruption by trypsin treatment of cells. Trypsin-treated keratinocytes exhibited β -catenin degradation (Fig. 6A). Concomitantly, ROS was highly increased (Fig. 6B), caspase 3 was activated (Fig. 6A) and cells died (Fig. 6C). These results indicate that disruption of cell adhesion can initiate the ROS-caspase- β -catenin degradation killing loop. Staurosporine, a non-specific kinase inhibitor, impairs mitochondrial function and is commonly used as an inducer of apoptosis (Tafani et al, 2002; Tafani et al, 2001). We treated wild type keratinocytes with staurosporine and observed that it induced β -catenin degradation, caspase activation, ROS accumulation and cell death (Fig. 6D–F). Staurosporine-induced β -catenin degradation and caspase activation was blocked by BHA treatment (supplementary Fig. S4). Thus, staurosporin can activate the ROS-caspase- β -catenin degradation loop. To determine whether this loop mediates cell killing in other epithelial cells, we analyzed human embryonic kidney 293 cells stimulated with combination of TNF-related apoptosis inducing ligand (TRAIL) and cycloheximide, which is an inducer of apoptosis in 293 cells. We detected cleavage of β -catenins and observed a moderate increase of caspases 3 activation and ROS accumulation (Fig. 6G–I). Collectively, these results support the idea that there is a ROS-caspase- β -catenin degradation loop, which functions to kill epithelial cells (Fig. 7). ROS are known to damage mitochondria and promote caspase activation (Lin & Beal, 2006). When ROS are accumulated and damage mitochondria or when cell adhesion is disrupted, the feed-forward loop consisting of ROS, caspases and β -catenin degradation, is activated and irreversibly kills the damaged epidermal cells. This killing loop is likely to be important for securely eliminating damaged epithelial cells, promoting repair, and preventing potential carcinogenesis in the epidermis.

Materials and Methods

Mice

tak1-floxed (*tak1*^{flox/flox}) mice were from Dr. Akira, Osaka University (Sato et al, 2005). Mice harboring an epidermal-specific constitutive *tak1* deletion (*K5-Cre tak1*^{flox/flox}) were generated using *K5-Cre* mice (mixed background of C57LBL/6 and DBA/2J)2. *K14-CreERT* mice were obtained from the Jackson Labs (Vasioukhin et al, 1999). Mice harboring an epidermal-specific inducible *tak1* deletion (*K14-CreERT tak1*^{flox/flox}) were generated and at 5 weeks of age were topically treated on the dorsal skin once a day for 5 consecutive days with 4-hydroxy tamoxifen (4-OHT) (1 mg/ mouse) (Omori et al, 2008). Some mice were fed with food containing 0.7 % BHA starting at 3 days prior to the tamoxifen treatment. *tak1* gene deletion was examined by PCR described previously. Littermate controls were used in all experiments. All animal experiments were done with the approval of the North Carolina State University Institutional Animal Care and Use Committee.

Cell culture

tak1 ^{+/+} and *tak1* ^{-/-} keratinocytes were isolated from *tak1* ^{flox/flox}, *K5-Cre tak1* ^{flox/flox} mice described previously (Omori et al, 2006). Spontaneously immortalized keratinocytes derived from the skin of postnatal day 0–2 mice were cultured in Ca²⁺-free minimal essential medium (Lonza) supplemented with 4% Chelex-treated bovine growth serum (Hyclone), 10 ng/ml of human or murine epidermal growth factor (Peprotech), 0.05 mM calcium chloride, and penicillin-streptomycin at 33°C in 8% CO₂.

Reagents

Reagents used were TNF- α (Peprotech), butylated hydroxyanisole (BHA), lithium chloride, 4-hydroxy tamoxifen (Sigma), MG132, Z-VAD-fmk, Staurosporine ALLM (Calbiochem), clasto-lactacystin β -lactone (Cayman), TRAIL (Peprotech) and cycloheximide (Merck). Polyclonal antibodies were caspase 3 and cleaved caspase 3 (Cell Signaling). Monoclonal antibodies were β -catenin, p120-catenin (BD), β -actin (Sigma) and E-cadherin (ZYMED). Alexa Fluor 488 phalloidin (Invitrogen) was used.

Immunoblotting

Keratinocytes were washed once with ice-cold phosphate-buffered saline and whole cell extracts were prepared using a lysis buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β -glycerophosphate, 1.5 mM MgCl₂, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 20 μ M aprotinin, 0.5% Triton X-100). Cell extracts were resolved on SDS-PAGE and transferred to Hybond-P membranes (GE Healthcare). The membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using the ECL or ECL advance Western blotting detection kit (GE Healthcare).

Immunohistochemistry

Bouin-fixed paraffin sections were heated in 10 mM citrate buffer for 10 min and β -catenin immunofluorescence staining was performed. dUTP nick-end labeling (TUNEL) assay was performed on Bouin-fixed paraffin sections using an apoptotic cell death detection kit (Promega) according to the manufacturer's instructions. Sections were counterstained with DAPI.

Cell viability assay

To determine the number of viable cells, a modified version of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium Bromide (MTT) assay, MTS assay, was performed according to the manufacturer's instructions (Promega).

Retroviral infection

Retroviral vectors for TAK1 (pMX-puro-TAK1) was described previously (Kim et al, 2008). β -catenins (pMX-puro- β -catenin WT and S33Y) were generated by inserting β -catenin cDNA into the retroviral vector which was generated from pMX-puro (Kitamura, 1998). β -catenin cDNAs (Kolligs et al, 1999) were gifts from Dr. Eric R. Fearon, The

University of Michigan Health System. EcoPack293 cells (BD) were transiently transfected with the various β -catenin vectors. After 48 h culture, growth medium containing retrovirus was collected and filtered with 0.45 μ m cellulose acetate membrane to remove packaging cells. Keratinocytes were incubated with the collected virus-containing medium with 8 μ g/ml polybrene for 24 h. Uninfected cells were removed by puromycin selection.

ROS measurement

Cells were stimulated, and then incubated with 10 μ M CM-H₂DCFDA (Invitrogen) for 30 min at 37°C. Harvested cells were analyzed by flow cytometry and FlowJo software (Tree Star Inc). One representative data from 2–3 similar results were shown in each experiment.

Trypsin treatment

Keratinocytes were trypsinized and incubated in Ca²⁺-free minimal essential medium with no calcium on plates coated with poly-2-hydroxyethyl methacrylate (10 mg/ml, Sigma).

Annexin V-binding assay

To determine apoptotic cells, Annexin V-Alexa Fluor 488 binding was performed according to the manufacturer's protocol (Invitrogen), and fluorescence was detected with fluorescent microscope (Olympus). 5–6 randomly selected areas were photographed with the same exposure time. The images were processed using the same fixed threshold in all samples by Photoshop software, and representative images are shown. The annexin positive cells were counted in total at least 1000 cells in each treatment, and one representative data with 2–3 similar results are shown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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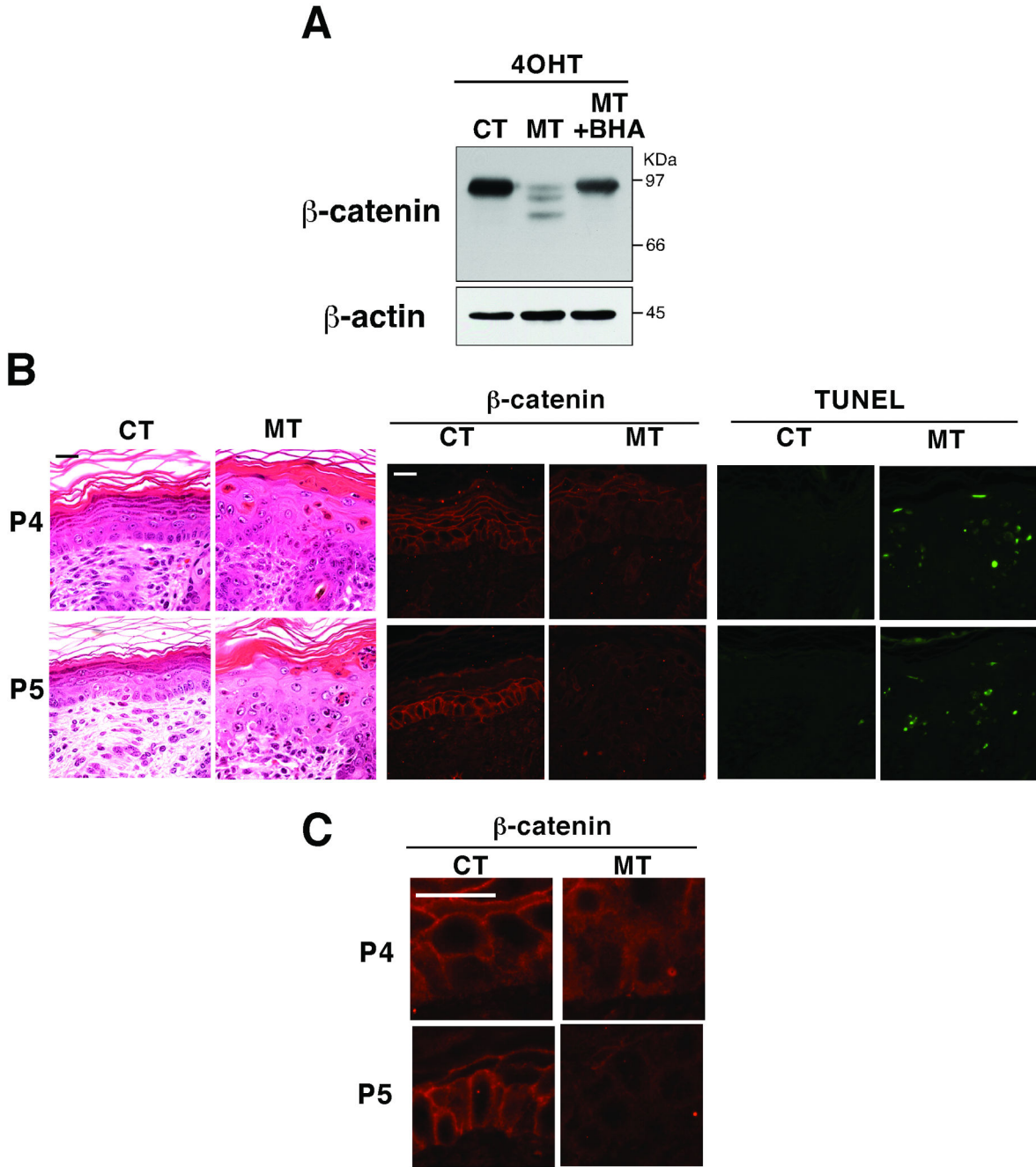


Fig. 1. (A) *tak1^{flox/flox}* (CT) and *K14-CreERT tak1^{flox/flox}* (MT) mice from the same litter were fed with BHA-containing or regular food, and treated with 4-OHT for 5 consecutive days. β-catenin was detected by immunoblot using protein extracts from epidermis at 2 days after the completion of 4-OHT treatment. (B) The dorsal skin sections of *TAK1^{flox/flox}* (CT) and *K5-Cre tak1^{flox/flox}* (MT) mice from the same litter at postnatal day 4 and 5 were stained with hematoxylin/eosin, anti-β-catenin antibody or TUNEL. Scale bar, 20 μm. (C) Pictures with a higher magnification of (B). Scale bar, 20 μm.

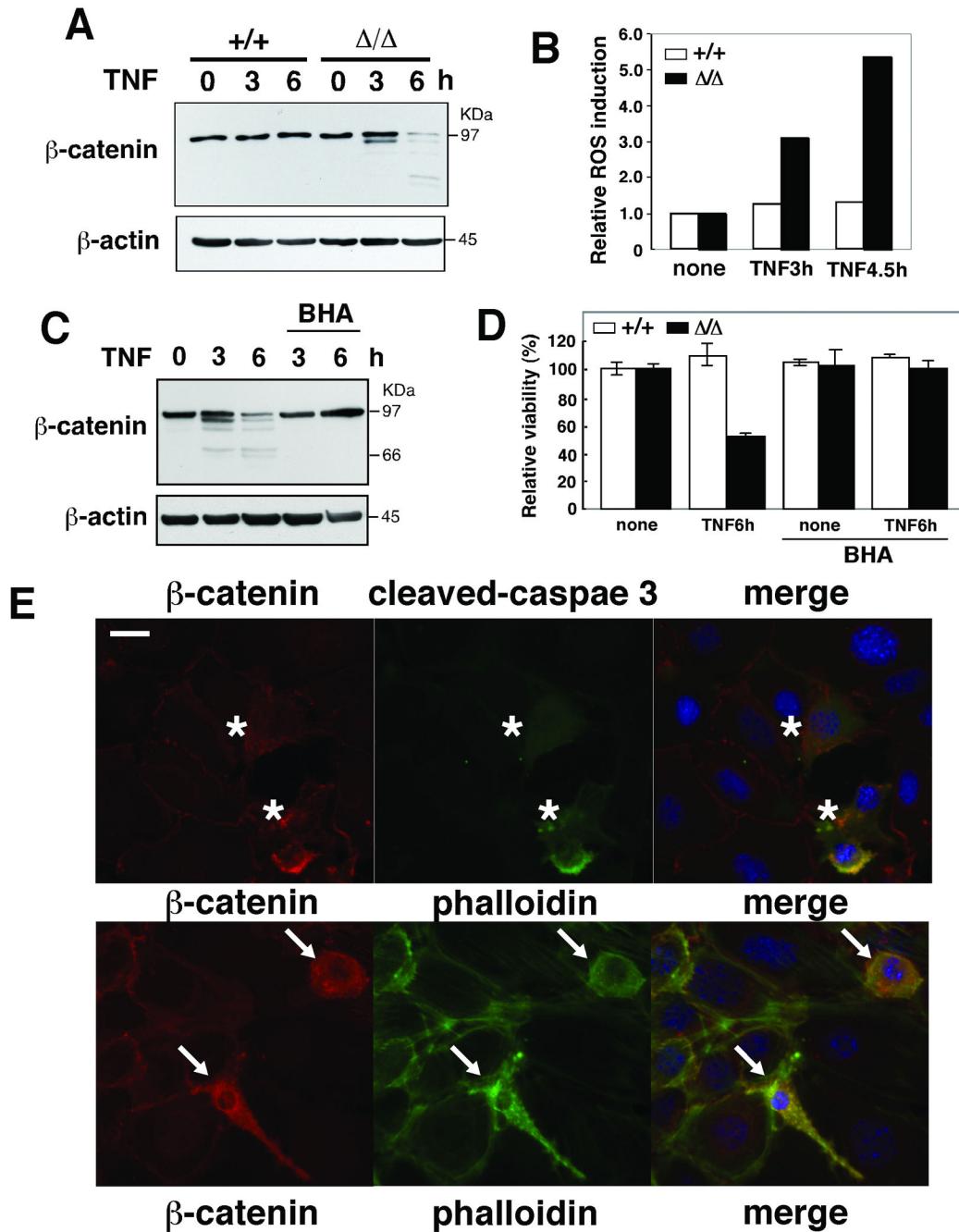


Fig. 2. (A) *tak1* wild-type (+/+) and *tak1*-deficient (/) keratinocytes were stimulated with 20 ng/ml TNF for 3 or 6 h and β-catenin was detected by immunoblot. (B) Cells with TNF stimulation were subsequently incubated with 10 μM CM-H2DCFDA for 30 min, and cells were analyzed by flow cytometry. The fluorescence units relative to that of unstimulated cells are shown. (C) *tak1* / keratinocytes were pretreated with 100 μM BHA or vehicle ethanol for 1 h and stimulated with 20 ng/ml TNF for 3 or 6 h. β-catenin was detected by immunoblot. (D) *tak1* +/+ and / keratinocytes were pretreated with 100 μM BHA or

vehicle ethanol for 1 h and stimulated with 20 ng/ml TNF for 6 h. Cell viability was measured by MTT assay. Data are the mean \pm S.E. of three samples. (E) *tak1* / keratinocytes were cultured in calcium-containing media for 2 h and subsequently stimulated with 20 ng/ml TNF for 24 h. Immunostaining of β -catenin (red), cleaved caspase 3 (green) and phalloidin (green) are shown. DAPI staining is shown in blue. Scale bar, 20 μ m. The cells having diffuse β -catenin, punctate phalloidin and cleaved caspase 3 staining were counted in three independent samples. 97.9% \pm SD 1.3% of cells having diffuse β -catenin staining were co-stained with cleaved caspase 3. 100% of cells having punctate phalloidin staining were co-stained with cleaved caspase 3.

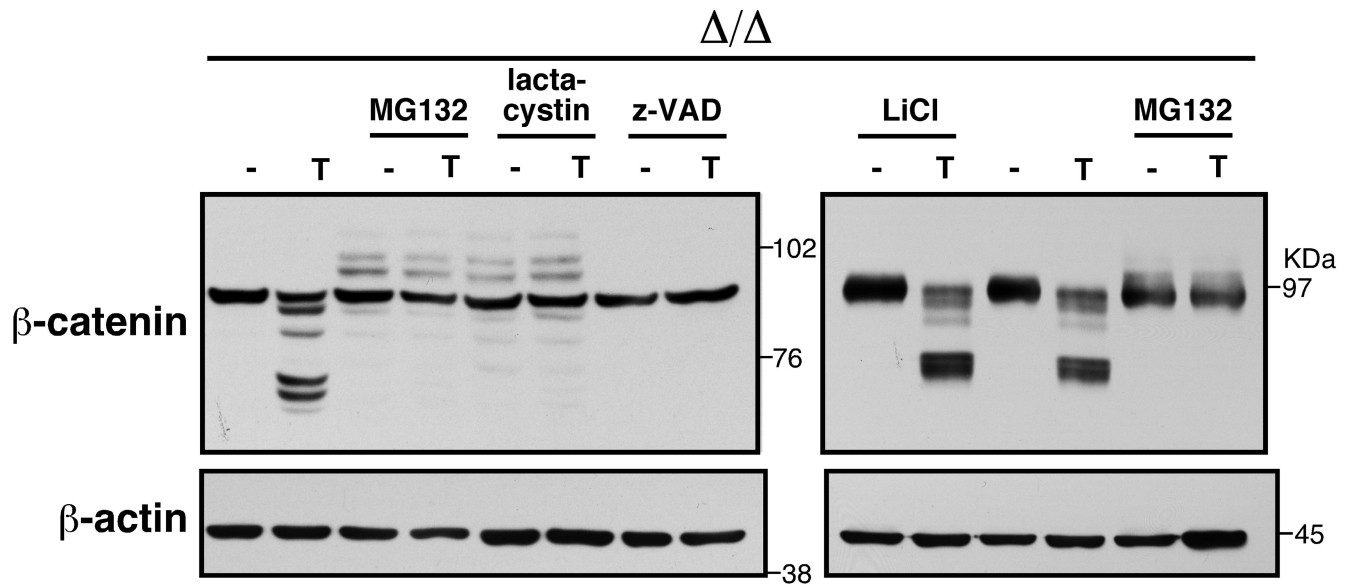


Fig. 3.
tak1^{-/-} keratinocytes were pretreated with the indicated inhibitors for 0.5 h (LiCl) or 1.0 h (other inhibitors) and stimulated with 20 ng/ml TNF for 4 h (left panels) or 4.5 h (right panels). Cell lysates were immunoblotted with anti- β -catenin. 10 μ M MG132, 5 μ M lactacystin, 20 μ M Z-VAD-fmk (Z-VAD), and 10 mM LiCl were used.

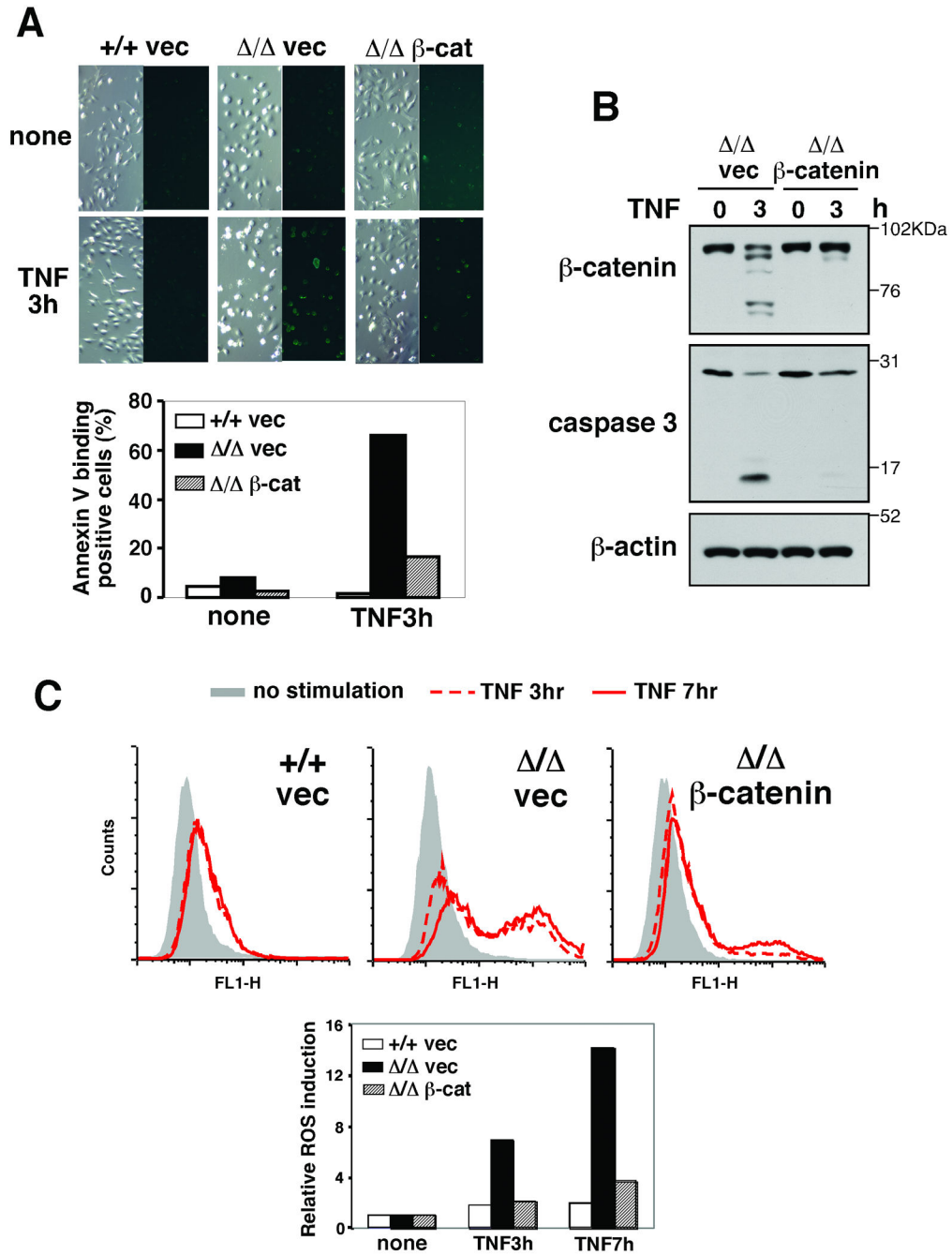


Fig. 4. (A) *tak1* $+/+$ and $-/-$ keratinocytes expressing vector or β -catenin were stimulated with 20 ng/ml TNF for 3 h and apoptotic cells were stained by Annexin V-Alexa Fluor 488. (B) *tak1* $-/-$ keratinocytes expressing vector or β -catenin were stimulated with 20 ng/ml TNF for 3 h and cell lysates were immunoblotted with the indicated antibodies. (C) *tak1* $+/+$ and $-/-$ keratinocytes expressing vector or β -catenin were stimulated with 20 ng/ml TNF for 3 or 7 h. Cells were subsequently incubated with 10 μ M CM- H_2 DCFDA for 30 min and analyzed

by flow cytometry. The fluorescence units relative to that of unstimulated cells are also shown.

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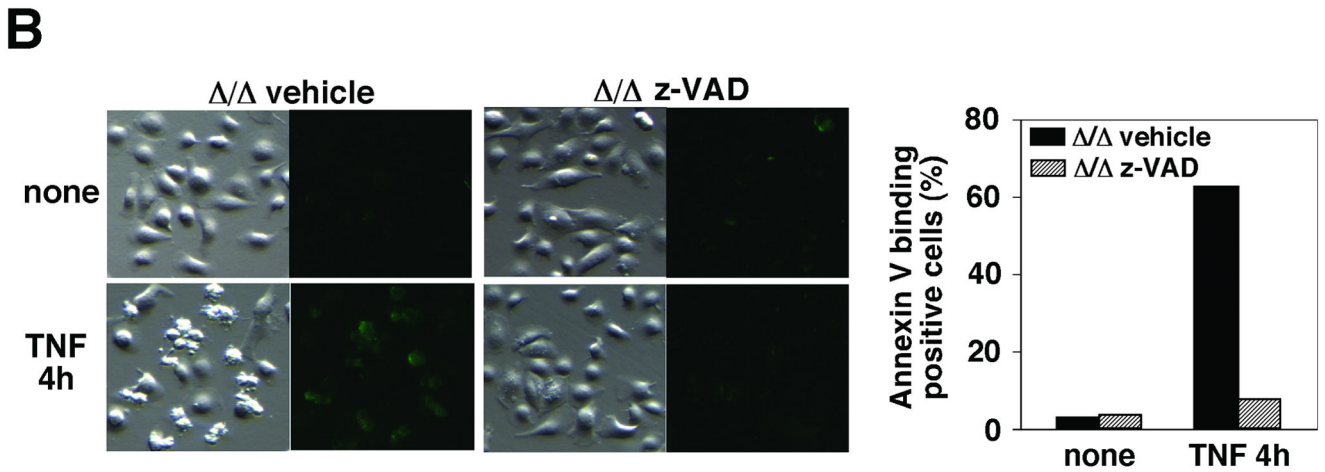
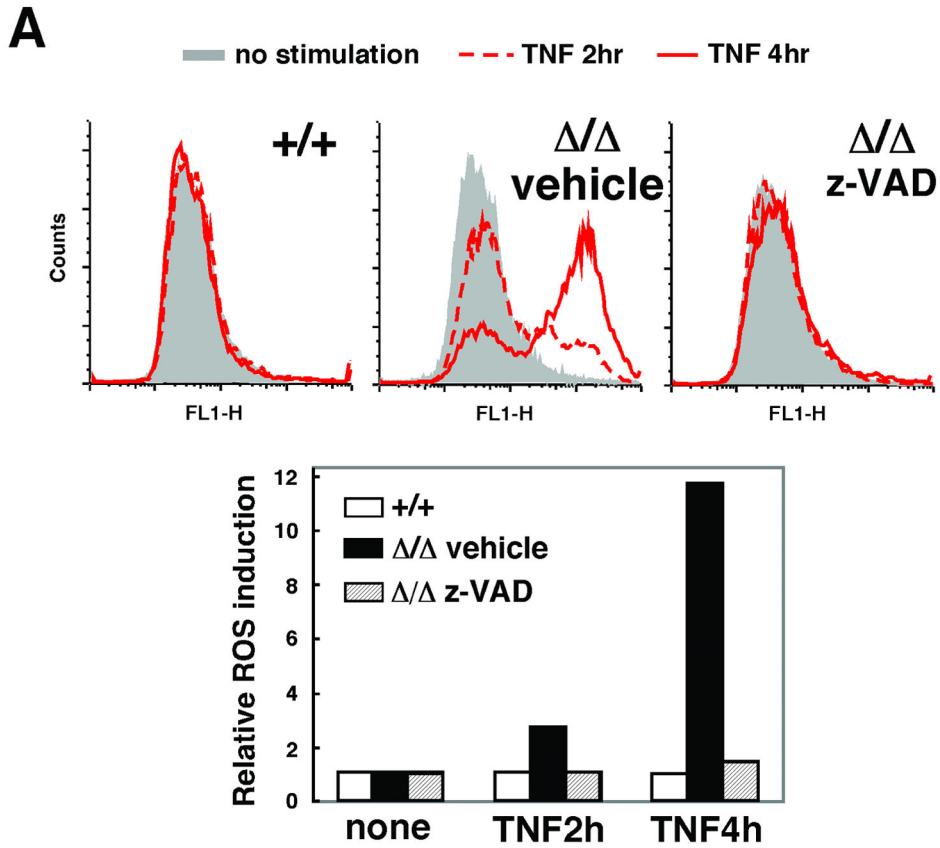
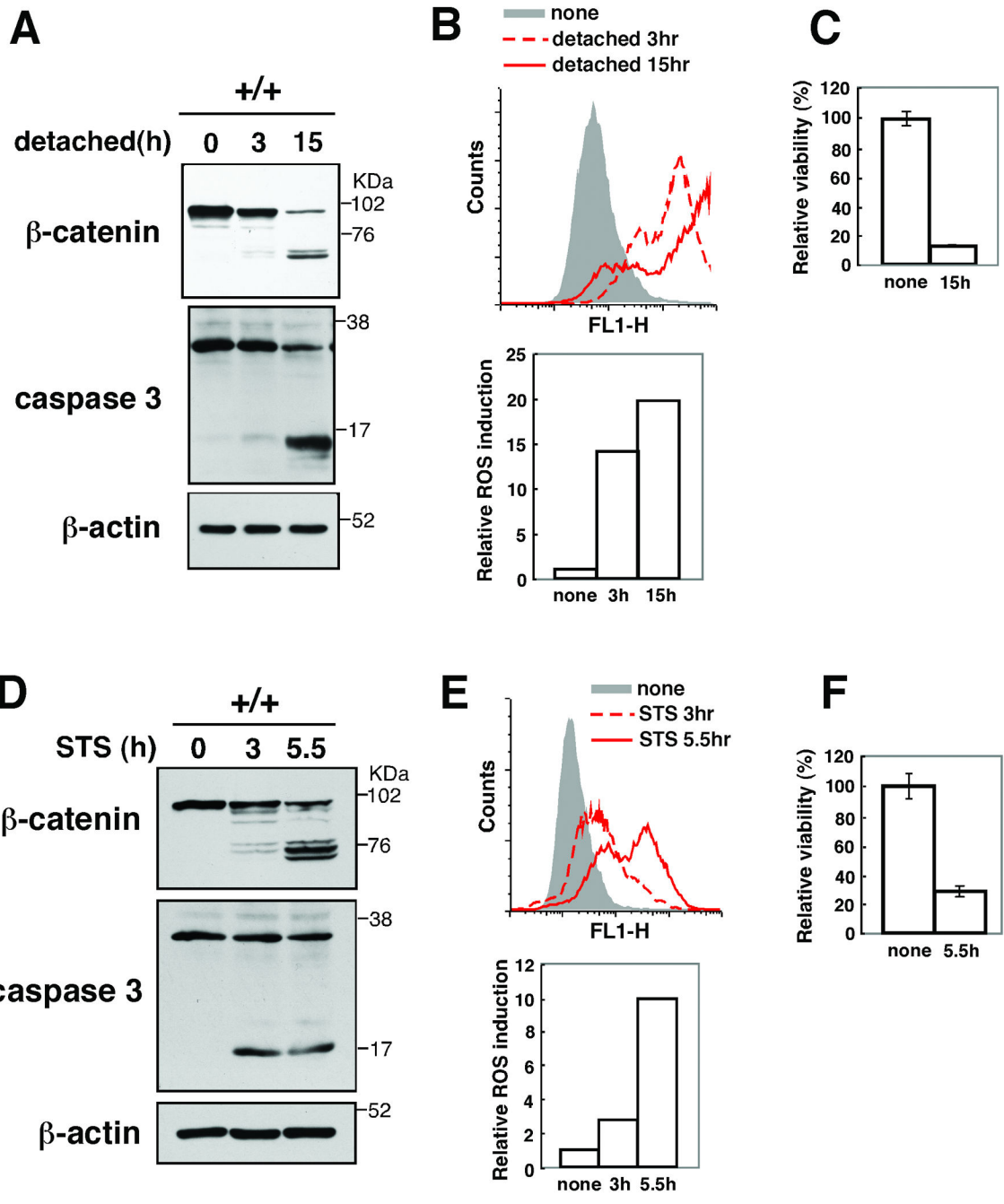


Fig. 5. (A) *tak1* $+/+$ and $-/-$ keratinocytes were pretreated with or without 20 mM Z-VAD-fmk or DMSO for 1 h and stimulated with 20 ng/ml TNF for 2 or 4 h. Cells were subsequently incubated with 10 μ M CM-H₂DCFDA for 30 min and analyzed by flow cytometry. The fluorescence units relative to that of unstimulated cells are also shown. (B) *tak1* $-/-$ keratinocytes were pretreated with 20 mM Z-VAD-fmk or DMSO for 1 h and stimulated with 20 ng/ml TNF for 4 h and apoptotic cells were stained by Annexin V-Alexa Fluor 488.



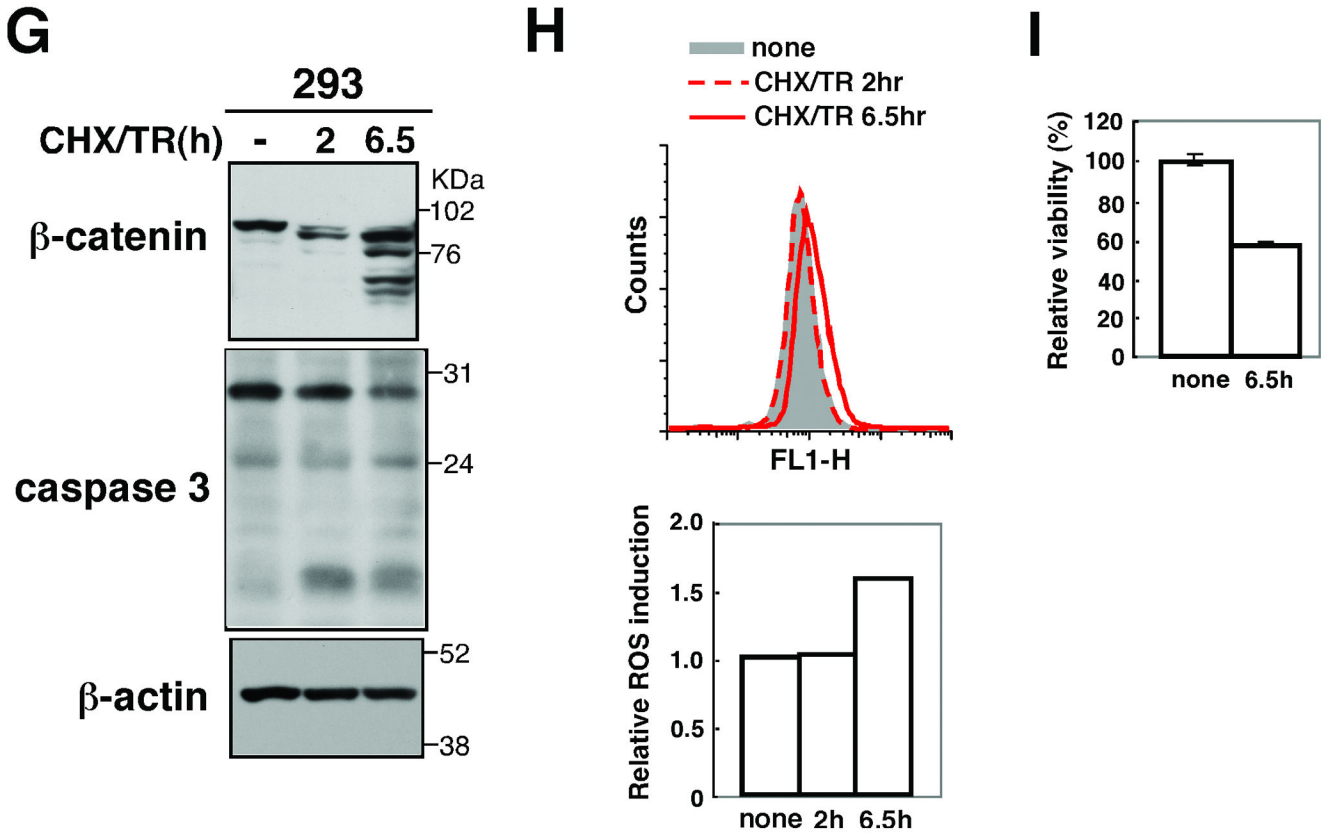


Fig. 6. (A–C) *tak1* *+/+* keratinocytes were trypsinized and cultured in calcium free media for 3 or 15 h. Cell lysates were immunoblotted with anti- β -catenin and anti-caspase 3 (A). Cells were incubated with 10 μ M CM-H₂DCFDA for 30 min and analyzed by flow cytometry (B). The fluorescence units relative to that of unstimulated cells are also shown (A, bottom). Cell viability was measured by MTT assay. Data are the mean \pm S.E. of three samples (C). (D–F) *tak1* *+/+* keratinocytes were stimulated with 0.5 μ M staurosporine (STS) for 3 or 5.5 h. Cells were analyzed as described above. (G–I) HEK293 cells were prestimulated with 100 μ g/ml cycloheximide (CHX) for 1 h and stimulated with 100 ng/ml TRAIL (TR) for 2 or 6.5 h. Cells were analyzed as described above.

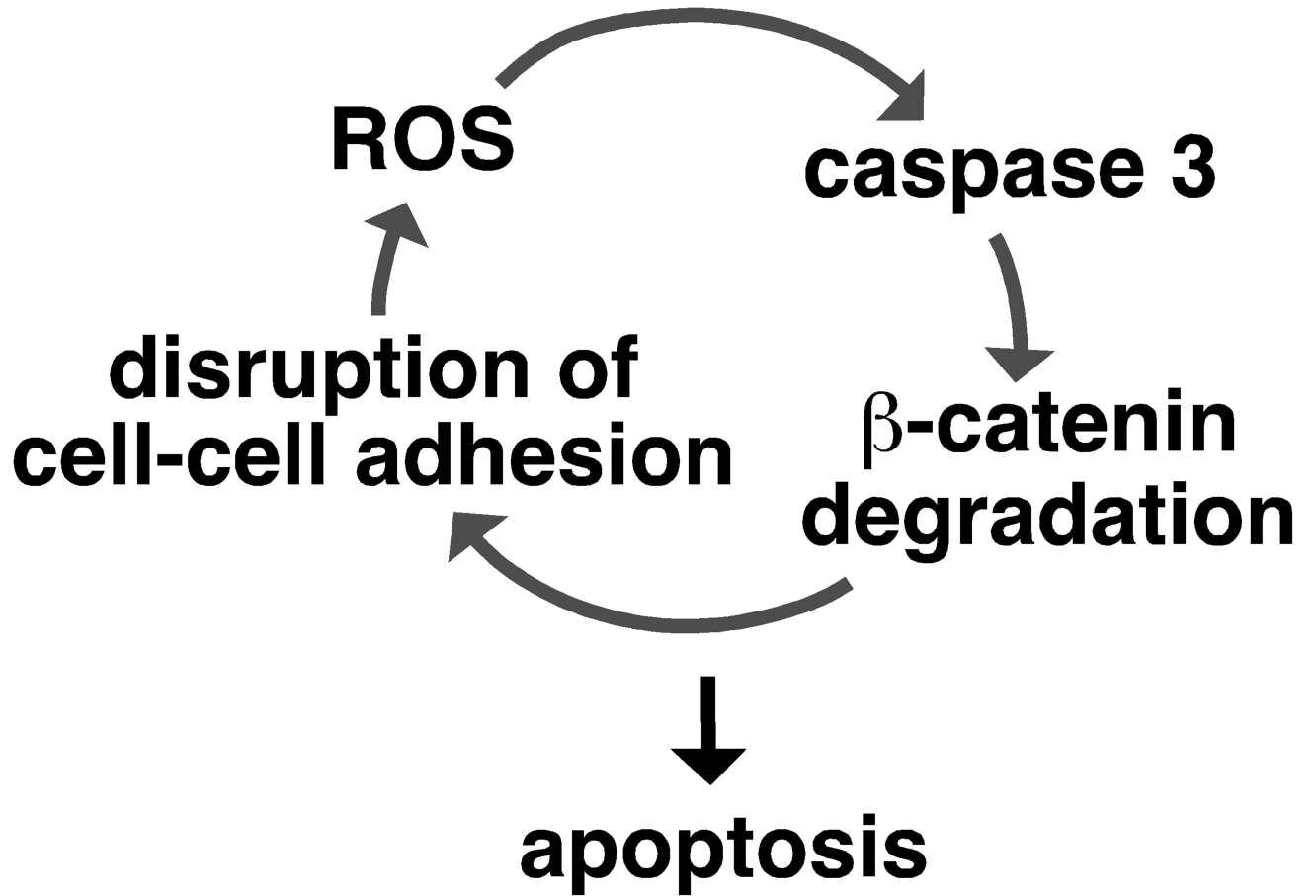


Fig. 7.
Model