Mixed-Lineage kinase-3 stabilizes and functionally cooperates with tribbles 3 to compromise mitochondrial integrity in cytokine-induced death of pancreatic beta cells.

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Running Head: MLK3 and TRB3 induce beta cell death via Bax.

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Mixed lineage kinases (MLKs) have been implicated in cytokine signaling, as well as in cell death pathways. Our studies show that MLK3 is activated in leukocyte-infiltrated islets of non-obese diabetic mice and that MLK3 activation compromises mitochondrial integrity and induces apoptosis of beta cells. Using an ex-vivo model of islet-splenocyte co-culture, we show that MLK3 mediates its effects via the pseudokinase TRB3, a mammalian homolog of drosophila tribbles. TRB3 expression strongly coincided with conformational change and mitochondrial translocation of BAX. Mechanistically, MLK3 directly interacted with and stabilized TRB3, resulting in inhibition of Akt, a strong suppressor of BAX translocation and mitochondrial membrane permeabilization. Accordingly, attenuation of MLK3 or TRB3 expression, each prevented cytokine-induced BAX conformational change and attenuated the progression to apoptosis. We conclude that MLKs compromise mitochondrial integrity and suppress cellular survival mechanisms via TRB3-dependent inhibition of Akt.

In type 1 diabetes, the autoimmune destruction of pancreatic beta cells is driven by leukocyte infiltration and the damaging effects of locally secreted cytokines. Cytokines activate MAP kinases JNK and p38, via signaling modules that involve the sequential activation of a MAP3K, MAP2K, and MAPK, all scaffolded by a single protein (1). Existence of several families of MAP3Ks raises the possibility that each MAP3K may be activated by specific classes of stimuli. The serine-threonine MAP3K Mixed Lineage Kinase-3 (MLK3) is activated by cytokines (2,3), and assembles a signaling module consisting of MKK7, JNK and the scaffold protein JIP1 (4,5). Fibroblasts with a targeted deletion of either MKK7 or MLK3 are attenuated in their response to cytokines (6,7). Elevation of MLK3 has been linked to induction of apoptosis in neurons (8-10) and inhibition of MLKs can delay progression of neurodegenerative diseases (reviewed in reference 11, and studies quoted therein). The striking parallels between the beta cell and neuronal phenotypes, coupled with the ability of cytokines to activate MLK3, prompted us to examine whether MLKs participate in cytokine-induced beta cell death.

Here we show that MLK3 is markedly elevated in leukocyte-infiltrated islets of the non-obese (NOD) type 1 diabetic mouse. To investigate the potential role of MLK3 in beta cell death we devised an ex-vivo system for co-culture of primary islets with immune-activated splenocytes. Compared to static culture with purified cytokines, this system is likely to be more representative of the milieu encountered by islets in autoimmune diabetes. We observed rapid activation of MLK3, and MLK3 was required for cytokine-mediated apoptosis via BAX, a proapoptotic member of the BCL-2 protein family. MLK3 mediated its effects via the pseudokinase TRB3, originally identified as an inducible factor in neuronal cell death (12), and subsequently shown to be a potent negative regulator of the pro-survival kinase Akt (13). We found that MLK3-mediated stabilization of TRB3, lead to a conformational change of BAX and the ensuing mitochondrial outer membrane permeabilization (MOMP) (14-16). For the vast majority of cells, MOMP has been identified as the commitment point for cellular apoptosis (17,18). In our studies, the strong correlation between the induction of MLK3 and TRB3, with
BAX conformational change, suggests that MLK3 activation may play a central role in committing the beta cell to an apoptotic fate.

**EXPERIMENTAL PROCEDURES**

**Reagents:** Antibodies used include mouse anti-Porin (MitoSciences, Eugene, OR), beta-tubulin, and HA (Covance, Berkeley, CA); rabbit anti-MLK3, pMLK3, BAX, Cytochrome C, JNK, pJNK, GST, AKT, phospho AKT, Myc, Flag (Cell Signaling, Beverly, MA), MLK1 and MLK2 (Abgent, San Diego, CA), and PDX-1 (19) for western blotting; Sheep anti-insulin (The Binding Site, San Diego, CA), mouse anti-BAX clone 6A7 (BD Bioscience, San Jose, CA), caspase-6 (20), TRB3 (Marc Montminy, Salk Institute CA), MLK3 (A. Rana, Loyola Univ IL) and pMLK3 (Cell Signaling, Beverly, CA) were also used for immunostaining after standardizing in cell lines (Fig S7). Other antibodies used to screen the SICC arrays were caspase-3, 7, 8 and 9 (20), Smarc and Traf3 (21), p53 and Fas (22), Bel-X<sub>S</sub>, BAK, tBid, Bid and BAD (20). Mouse anti-HA agarose beads (Sigma, St Louis, MO) and Myc agarose beads (Santa Cruz Biotech, Santa Cruz, CA); Glutathione-Sepharose beads (Amersham-Pharmacia, Piscataway, NJ) were used for immunoprecipitation and pulldown experiments. Fluorescence and western blotting employed fluorescent or HRP-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA), the latter detected using Supersignal chemiluminescence reagents (Pierce Biotechnology Inc, Rockford IL). Other reagents include In Situ TUNEL kit (Roche, Indianapolis, IN), MitoTraker and JC-1 dye (Invitrogen, Carlsbad, CA), CEP11004 (Cephalon Inc, Frazer, PA), Jnk-II inhibitor SP600125, cycloheximide (EMD biosciences, San Diego, CA), hIL-1β, hTNF-α, mIFN-γ and mIL-2 (Peprotech, Rocky Hill, NJ).

**Plasmids, Constructs and siRNA:** PEBG-MLK3 plasmids were a gift from Dr. Ajay Rana and TRB3 deletion constructs have been described elsewhere (23). The MLK3 and GL3-luciferase shRNA sequences GGCGACGCGATGTCTGGAGC (24) and CTTACGCCTGACTTCGCA, were used to generate a double stranded ology that was subcloned downstream of the U6 promoter in bluescript vector pBS-U6. pcDNA3-RFP-BAX was generated by amplifying BAX cDNA from CMVSport6 BAX (OpenBiosystems, Huntsville, AL) and subcloned in frame with RFP into pcDNA3-dsRED (Invitrogen). Myc-Akt was generated by PCR-subcloning Akt2 (Addgene) into EcoRI site of pcDNA3Myc (Stratagene) and sequence verified. CMVSport6 expression vectors for ASK1 and TAK1 were from Open Biosystems, pcDNA3-MEK1 was a gift from Dr. R. Davis, U. Mass. MA, and pcDNA3-TPL2, was a gift from P. Tsichlis, Tufts Univ. MA. For bacterial expression vectors, BamH1 digests of full-length wildtype and kinase-dead MLK3 from PEBG expression vectors were subcloned in-frame into PGEX-4T1 vector.

**Generation of recombinant Akt adenovirus:** Myristlated-AKT (T308D/S473D) and AKT-KD (K179M) were subcloned into pAdTrack-CMV and adenoviruses were generated using the AdEasy system as previously described (25).

**Splenocyte and Islet Co-Culture (SICC) and SICC-Cell Array:** Diabetic NOD spleens were crushed and passed through a 70µm mesh. Red blood cells were lysed in 0.15M NH₄Cl and 1.5 X 10⁶ splenocytes per well were plated in 24 well dishes in RPMI supplemented with 60U/ml of mIL-2 and 10% heat-inactivated FBS. Splenocytes were stimulated with plate bound anti-CD3 and exogenous anti-CD28 antibodies (10µg/ml and 1µg/ml respectively; BD Biosc, San Diego, CA) for 3 days as previously described (26). Islets were isolated 2 days later, rested overnight, and cultured with transwell filters in the presence of unstimulated or stimulated splenocytes, with or without 500nM CEP11004 pretreatment, and collected over a time-course of 24 hours. At each time-point, islets were fixed, spun into pellets, and embedded in agarose plugs. Individual plugs from an entire time-course were laid out on a grid, and processed for paraffin embedding in a single block as described (27).

**Tissue Preparation, Immunohistochemistry and Immunofluorescence:** Isolated islets and pancreata were fixed in Bouins Fixative for 30mins and 4 hours respectively, washed in PBS, post-fixed in Z-Fix (Anatech, Battle Hill, MI), processed for paraffin embedding and immununostained as previously described (20,27,28). For staining, slides were pretreated with targeted retrieval solution, high pH (DAKO, Carpinteria, CA), as per manufacturers instructions or in 110mM citrate buffer, pH 6.0 and primary antibodies were revealed using a dianaminobenzidine (DAB) based detection method employing either an avidin–biotin complex reagent (Vector Laboratories, Burlingame, CA) or the Envision-plus-horse...
radish peroxidase (HRP) system (DAKO, Carpinteria, CA). For immunofluorescence, Min6 cells were fixed in 4% paraformaldehyde for 5 mins, permeabilized with 0.1% triton-X and primary antibodies were visualized with species-specific secondary antibodies conjugated to fluorescent probes. **Human adult islets:** Human adult islets were provided by Islet Cell Resource Center Basic Science Human Islet Distribution Program and the Islet Transplant Program, University of Illinois Chicago. Islets were hand picked and cultured overnight in CRML supplemented with 10% FBS. Prior to stimulation with cytokines islets were cultured for at least 8 hours in RPMI-1640 supplemented with 10% FBS. **Mouse Islet Isolation:** Islets were isolated using a ficoll gradient as described (29). **TUNEL Assay:** Dewaxed sections of the SICC array were pre-treated with 10µg/mL Proteinase K for 30mins at 37°C, while Min6 cells were fixed in 2% paraformaldehyde prior to TUNEL labeling as per manufacturers instructions (Roche, Indianapolis, IN). **Mitochondrial Potential:** For confocal microscopy, cells were incubated with JC-1 (2.5µg/mL) for 15min at 37°C following the indicated treatments and examined immediately. For quantification, cells were processed using the Guava MitoPotential Kit. Cell populations were gated based on JC-1 fluorescence and quantified using the Guava EasyCyte Flow Cytometer (Guava Tech., Hayward, CA). **Mitochondrial Fractionation:** Using a modified method (30), cells were harvested and allowed to swell for 30 min in hypotonic buffer (10mM Hapes, 5mM MgCl2, 40mMKCl, 10µg/ml aprotinin and leupeptin). After 30 passes through a 25 gauge needle, sucrose was added to a final conc. of 250 mM, along with Na Orthovanadate (0.5 mM), and PMSF (1 mM), nuclei and cell debris removed by centrifugation at 800x g for 10 min at 4°C. The supernatant was centrifuged at 10,000xg for 20 min at 4°C to separate mitochondrial and cytosolic fractions, and 15µg protein was used for SDS-PAGE. **Cell Culture, Transfection, Infection and FACS sorting:** Min6 cells (passages 15-18 only) were grown in DMEM containing 25mM glucose supplemented with 4% heat inactivated FBS and 50µM β-mercaptoethanol. HEPG2 cells were grown in a 1:1 mix of DMEM and F12K and 5% heat inactivated FBS. Cos-7 cells were grown in DMEM and 10% heat inactivated FBS. Transfections for GST pulldown assays, immunoprecipitation and BAX-RFP translocation in Min6, HEPG2 and Cos-7 cells were performed using Lipofectamine 2000 (Invitrogen) as per manufacturer’s instructions and were treated as described 48 hours post transfection. Min6 cells were infected with Ad Akt-Myr or AdAkt-KD (MOI=25) for 6 hours in growth medium and 40 hours post-infection were treated as described. For endogenous MLK3 knockdown, due to the low transfection efficiency of β cell lines, Beta TC-3 cells were nucleofected with U6 shRNA constructs and pcDNA3-RFP reporter construct, used as a co-transfection marker. Cell sorting (FACS-DiVa, BD Biosciences San Jose, CA) gated for RFP expression, was followed by western blotting analysis of MLK3 protein. **Nucleofection of shRNA constructs and transfection of siRNA:** For shRNA rescue experiments, 1X10^6 Min6 cells were electroporated using Nucleofector Kit V (Amaxa GmbH, Cologna, Germany) with 2µg of plasmid DNA encoding shRNA or MLK3-KD, and 0.4µg of pcDNA3-RFP. Indicated treatments were performed 40-48 hours post-nucleofection. **BAX-RFP Translocation:** Cos-7 cells transfected with pcDNA3-RFP, pcDNA3-BAX-RFP, pcDNA3, pcDNA3-HA-TRB3 as indicated, were incubated with mitotracker dye (200nM) for 30mins at 37°C, fixed and analyzed immediately. **Cycloheximide chase, western blotting, and insulin stimulation:** These experiments were performed as described (29). **GST-pulldown assay and immunoprecipitation:** Mammalian expression vectors encoding GST or HA- or Myc-tagged fusion proteins were expressed in Min6 or HepG2 cells followed by GST pulldowns with Glutathione Sepharose (Amersham-Pharmacia, Piscataway, NJ), or immunoprecipitations as described (29). **In vitro protein interactions:** Bacterial MLK3 proteins were expressed using BL21-RIL cells (Stratagene, La Jolla CA) and purified with Glutathione-Sepharose beads (Amersham-Pharmacia, Piscataway, NJ) according to the manufacturer’s instructions. TRB3 protein was generated by the TNT- coupled reticulocyte lysate system (Promega Corp. Madison WI) using T7 polymerase, and pcDNA3-HA-TRB3 as template with a full complement of amino acids. Glutathione sepharose bound GST-MLK3 WT or KD proteins were incubated with equal amounts of in vitro synthesized TRB3 protein in NETN buffer and full complement of protease and phosphatase...
inhibitors. The GST pulldowns were washed using NETN, and subjected to SDS-PAGE and the top and bottom halves of the transferred blots probed using pMLK3 (125kd) and TRB3 (48kd) antibodies respectively. MLK3 input was detected using Gel-Code Blue stain reagent (Thermo-Pierce).

**Microscopy and Image Acquisition:** Fluorescent and Brightfield images were acquired on either an inverted Olympus IX81 microscope attached to the Radiance 2100MP laser scanning system (Bio-Rad, Hercules, CA) or a ScanScope CS digital slide scanner (Aperio Technologies, Vista, CA) respectively. All images were assembled in Photoshop 10 (Adobe Systems Inc, San Jose, CA).

**Statistics:** Quantification was performed on ten randomly selected fields of view using the 40X or 60X objective from 3 separate experiments. Total cell counts were obtained using propidium iodide (0.05µg/ml), DAPI (1µg/ml), RFP, GFP, or insulin for cell specificity in SICC arrays. Quantification of caspase-6 was performed on images deconvolved using ImageScope software (Aperio Technologies, Vista, CA) and the caspase-6 positive pixels were expressed as a percentage of total pixels. Differences between means were examined using analysis of variance (ANOVA) followed by a Bonferroni post hoc comparison. In all cases, P values less than or equal to 0.05 were considered significant. Analysis was performed using StatView 5.0 statistical software.

**RESULTS**

**Cytokines induce expression of MLK3 in the pancreatic beta cell:** NOD mice spontaneously develop autoimmune diabetes around 15-18 weeks of age. Pancreas sections from 6 or 18-week old NOD female mice with respective ambient glucose levels of 110 and 280 mgs/dl, were stained for MLK3 and its active form (pMLK3). Both total and pMLK3 were strongly induced in leukocyte-infiltrated islets (Fig 1A), but not detected in uninfiltrated islets from the same mouse, or in islets of prediabetic NOD mice. These data suggested a correlation between local secretion of cytokines and induction and activation of MLK3. A widely used cocktail of cytokines for study of beta cell death includes the three components IL-1β, TNF-α and IFN-γ (31). Using each cytokine to stimulate Min6 cells, we found that in the beta cell, IL-1β rapidly induced total MLK3 protein levels within 20-30 minutes (Fig 1B). Similarly, in human islets, IL-1β acutely induced the MLK3 isoform of the mixed lineage kinases by more than four-fold. A smaller effect on MLK1 and 2 (Fig S1) was observed in human islets. Of note, the rapid upregulation of MLK3 protein was not due to increased transcription as no increase in MLK3 mRNA was observed (Fig S2). Our data mirrored a key finding in a previous report where apoptogenic stimuli were shown to rapidly upregulate MLK3 protein (32) by increasing protein stability.

Since MLK3 is a MAP3K, which preferentially activates JNK (33), we examined whether IL-1β induced JNK activity in an MLK3-dependent manner. Min6 insulinoma cells were treated with IL-1β, in the presence and absence of 500nM CEP11004, a potent inhibitor of the mixed lineage kinase family (34), whose specificity was first ascertained as shown (Fig S3). IL-1β rapidly induced JNK activation in the beta cell, and this effect was strongly attenuated by pretreatment with CEP11004 (Fig 1C).

To demonstrate cytokine-mediated induction of MLK3 in a physiologically relevant system, we devised an ex-vivo splenocyte-islet co-culture (SICC) system, which would mimic the islet response to leukocyte invasion in insulitis. Unlike the events in vivo, this system (depicted by a schematic in Fig 1D), permits the study of the earliest molecular events in cytokine signaling in a relatively controlled environment, while maintaining the complex, cytokine environment of insulitis. Splenocytes from diabetic NOD mice were isolated, and stimulated with anti-CD3 and anti-CD28 antibodies to trigger production of the full repertoire of cytokines (Table ST1). During co-culture, islets were suspended in transwells such that they were immersed in stimulated medium, but had no direct contact with splenocytes. To study the effect of MLK3, islets were co-cultured with or without pretreatment with CEP11004. Again MLK3 was rapidly (30 mins) activated by cytokines and western blotting of islet extracts from SICC (Fig 1E) showed that increasing numbers of splenocytes induced MLK3 and activated JNK, in a dose dependent manner. MLK3 and JNK were both inhibited using CEP11004, strongly suggesting that similar to IL-β, conditions that mimic insulitis also activate MLK3 and its downstream kinase JNK.

**MLKs mediate cytokine-dependent beta cell death:** To examine whether MLKs were required for inducing autoimmune destruction of beta cells, we employed the splenocyte islet co-culture (SICC) system described above. A time-course of SICC
islets from all experimental groups was arrayed on a single slide to enable a clear comparison across various samples. Similar to data from western blotting, (Fig 1E), MLK3 induction was also detected by immunostaining, and pretreatment with CEP11004 markedly inhibited this induction (Fig 2A, column 1, rows ii-iii). Within 24 hrs of co-culture, 15% of the insulin expressing cells in the islet were positive for TUNEL (column 2 row ii) which detects DNA strand breaks in end stage cell-death. Pretreatment with CEP11004, effectively reduced TUNEL staining to unstimulated levels (column 2 row iii). CEP11004 treatment similarly inhibited the cleavage and activation of caspase-6 (Fig S4). Quantification of TUNEL is shown in graph format (2B).

**TRB3 and BAX are downstream effectors of MLK3**: The arrayed time course of SICC islets was screened for shifts in expression of several apoptosis markers (see methods). Distinct MLK-dependent induction was observed for only two markers. The first was the pseudokinase TRB3 (Fig 2A column 3), which has been implicated in stress-induced apoptosis (12,35,36). The second marker was BAX, (Fig 2A column 4) a pro-apoptotic member of the BCL-2 family of proteins, known to be critically required for pancreatic beta cell apoptosis (37). Apoptotic events have been shown to trigger a conformational change in BAX, which marks a critical transition towards its insertion in the mitochondrial membrane to initiate apoptosis. This structural change exposes a stretch of seven amino acids, normally buried in a hydrophobic pocket under quiescent conditions (38). Using a monoclonal (6A7) antibody that specifically detects this exposed region, immunostaining of SICC islets (Fig 2B column 4) revealed intense punctate staining of BAX (row ii), and both TRB3 and BAX-6A7 induction were attenuated by pretreatment with the MLK inhibitor CEP11004 (row iii). Similar induction of TRB3 (Fig 2C panels i-ii) and BAX conformational change (panels iii-iv) were also detected in leukocyte-infiltrated islets of NOD mice.

**TRB3 induction, correlates with BAX translocation and a compromise in mitochondrial function**: To understand the mechanistic connection between MLKs, TRB3 and BAX, we used activated splenocyte-derived conditioned or ‘stimulated’ medium to treat Min6 mouse insulinoma cells followed by immunofluorescent staining at 8 hours (Fig 3A). For clarity, a cartoon outline of the stained cells is presented under each panel (bottom). As seen in Fig 3A (top) TRB3 was induced in 20% of Min6 cells treated with stimulated medium (Fig 3A top panel column 2). Remarkably, co-staining with 6A7 antibody detected conformationally-altered BAX in most TRB3 positive cells (column 3), with little signal in TRB3 negative cells (see bottom panel), suggesting a close relationship between TRB3 induction and BAX conformational change. Indeed, overexpression of TRB3 was sufficient to induce mitochondrial translocation of coexpressed BAX-RFP fusion protein in cultured Cos7 cells. As seen in Fig 3B, unlike the diffused localization of BAX-RFP in the presence of CMV control vector, presence of TRB3 resulted in a strong overlap of BAX-RFP signal with the green mitotracker dye. We next examined whether TRB3 protein induction was MLK-dependent. Using western blotting we found that stimulated medium (Fig 3C) and IL-1ß (Fig 3D), both strongly induced TRB3 protein and pretreatment with MLK inhibitor CEP11004, completely abrogated this effect. TRB3 protein induction was not accompanied by change in TRB3 mRNA (Fig S2), suggesting a post-transcriptional mechanism for TRB3 regulation.

To test whether the observed conformational change in BAX lead to membrane insertion and mitochondrial outer membrane permeabilization (MOMP), we examined levels of BAX in subcellular fractions from Min6 cells treated with stimulated medium for 8 hours. Western blots showed an increase in mitochondrial BAX and a corresponding reciprocal drop in cytoplasmic BAX (Fig 3D), as early as 4 hours and up to 12 hrs (not shown). In parallel, an inverse pattern of cytochrome C localization was observed, which indicated a breach in mitochondrial membrane integrity (39). Presence of CEP11004 inhibited both BAX translocation and Cytochrome C leaching, linking both these events to activation of MLKs. In the beta cell, mitochondria are central for glucose sensing and coupled insulin secretion. Since actively respiring cells have over 1000 mitochondria per cell, we examined whether the detected mitochondrial breach was sufficient to impact cellular respiration. In intact, actively respiring mitochondria, JC-1 dye forms j-aggregates that fluoresce red. Both confocal analysis and flow cytometry showed that an 8-16 hr incubation of Min6 cells with cytokine-rich stimulated medium resulted in ≥30% drop in red fluorescence, (Fig 3E...
panel ii & iv), which was almost completely reversed in the presence of CEP11004 (panel iii & iv).

In Min6 cells, CEP11004 treatment also efficiently inhibited stimulated medium mediated activation of caspase-6 (Fig S4) and end stage apoptosis measured by TUNEL. (Fig 3E, panels v-viii). Similar to data for primary islets, apoptosis induced by conditioned medium in Min6 cells was also inhibited by CEP11004 pretreatment. Overall, a contemporaneous induction and colocalization of endogenous TRB3 protein with BAX translocation, suggested a tight functional correlation between the two proteins, thus linking MLK3 and TRB3 to the mitochondrial death machinery via BAX.

**MLK3 and TRB3 knockdown inhibit BAX conformational change, and beta cell death:** Next we used shRNA directed against MLK3 (shRNA validation shown in Fig S5) and TRB3 (23), as an alternate means of inhibiting stimulated medium-induced apoptosis. Due to inefficient DNA uptake by Min6 cells, shRNA plasmids were cotransfected/nucleofected (Amaza Biosystems) with plasmid expressing CMV-red fluorescence protein (pseudocolored blue) to accurately track cells that took up shRNA (Fig 4A, column 1). Cells treated for 4-8 hours with unstimulated (row i) and stimulated medium (rows ii-iv) were immunostained using TRB3 (column 2, detected with Alexa-fluor-488 green) and conformation specific anti-Bax-6A7 (column 3, detected with Alexa-fluor-647, pseudocolored red) antibodies. For clarity, a cartoon of shRNA expressing cells in blue, and the TRB3 and BAX-6A7 double positive cells in yellow is shown in column 5. Induction of endogenous TRB3 protein again strongly coincided with BAX conformational change (denoted by arrowheads, and in yellow in column 5). Expression of control shRNA (U6-GL3, row i and ii) did not inhibit induction of 6A7-BAX and TRB3 (marked by arrow, row ii, column 4). Accordingly, as represented in column 5, TRB3 and BAX-6A7 double positive cells (yellow) showed a clear overlap with control plasmid expressing cells in blue. In contrast, both MLK3 (U6-MLK3, row iii) and TRB3 shRNA (U6-TRB3, row iv) attenuated induction of TRB3 and BAX conformational change as evidenced by the exclusion of BAX-6A7 and TRB3 from the shRNA-transfected, pseudocolored blue cells (column 4 and 5, rows iii & iv). The percent transfected (RFP+ pseudocolored blue) cells positive for co-expression of BAX-6A7 and TRB3, are shown in graphical format in Fig 4C.

Using the same approach as in Fig 4A, we examined whether knockdown of MLK3 and TRB3 also attenuated end-stage apoptosis. Min6 cells were nucleofected, using RFP to track the cells expressing shRNA constructs. After a 24-hour incubation with stimulated medium, transfected-RFP and TUNEL (green) double positive cells were quantified and expressed as a percentage of the total transfected cells. The percentage of untransfected TUNEL positive cells was comparable across treatments. Compared to U6-GL3 control (i) kinase-dead (KD) MLK3 (iii), or knockdown of either MLK3 (ii) or TRB3 (iv) all decreased apoptosis by $\approx 60\%$ (Fig 4B & D).

**MLK3 interacts with and stabilizes TRB3:** In the absence of a discernible effect of cytokine-dependent induction of TRB3 mRNA at time points when TRB3 protein induction was observed (Fig S2), we examined whether MLK3 associated with TRB3. Using bacterially expressed wildtype (WT) and kinase dead (KD) proteins, we show that GST-MLK3-WT interacted more avidly with in vitro transcribed and translated TRB3 compared to GST-MLK3-KD (Fig 5A, bottom panel compare lanes 3 and 4). Having observed that MLK3 could directly bind TRB3 in vitro, we cotransfected HepG2 cells with HA-TRB3 and a mammalian vector for GST-MLK3. Western blots of GST pulldowns (Fig 5B) showed that similar to the in vitro data, TRB3 interacted with GST-MLK3-WT and less avidly with GST-MLK3-KD (panel iii, compare lanes 2, and 5). Importantly, MLK3 protein levels appeared to correlate with its own phosphorylation status and a 60' exposure to CEP11004 abrogated phosphorylation and destabilized MLK3-WT (lanes 2-3, panels i & ii). Notably, total TRB3 protein levels (panel iv, compare lane 2 with lanes 3 and 5) tracked with MLK3 protein levels (panel ii), and as shown by others (32), catalytically active MLK3 (lanes 1-2) was more stable compared to catalytic inactivation of MLK3 using CEP11004 (lane 3), or using kinase dead MLK3-KD (lane 4-5). Co-transfected ß-tubulin control remained constant (panel iv).

To further qualify the interaction between MLK3 and TRB3, we sought to map the MLK3 interaction domain in TRB3. We cotransfected HepG2 cells with GST-MLK3-WT, and full-length or truncation mutants of TRB3 and analyzed the interaction using GST pulldown assays (Fig 5C). MLK3 was unable to bind N-terminal deletion mutants of TRB3 (lanes 2-3)
with interaction localized to the N-terminal 94 amino acids of TRB3. Divergence of the TRB3 N-terminus from other members of the tribbles family of proteins, attests to the likely specificity of the MLK3-TRB3 interaction.

In addition to demonstrating protein-protein interaction, data from Fig 5B suggested other attributes of MLK3-TRB3 interaction. Based on its ability to dimerize, catalytically active MLK3 has been shown to be more stable (32) as evidenced by higher levels of WT-MLK3 in Fig 5B. Interestingly, total TRB3 protein levels appeared to parallel those of MLK3 protein itself. Therefore, we hypothesized that binding MLK3 may result in increased TRB3 protein stability. We used cycloheximide chase experiments to determine the stability of HA-TRB3-WT, and MLK3 interaction-defective HA-TRB3-Δ94. As seen in Fig 5E and F, in presence of GST-MLK3-WT, the half-life (T½) of TRB3-WT was more than 3 hours, while T½ of HA-TRB3-Δ94 was sharply reduced to 45 minutes. These data showed that interaction with MLK3 increased the stability of TRB3. Knockdown of TRB3 rescues cytokine-mediated inhibition of Akt: Akt has been identified as a survival factor based on its ability to preserve mitochondrial integrity (40,41). Other studies have shown that Akt activity can be inhibited by proinflammatory cytokines (42,43). We therefore examined whether TRB3 knockdown attenuated the ability of cytokines to inhibit Akt Ser473-phosphorylation (Fig 6A) in Min6 cells. In the presence of control U6-GL3, insulin treatment activated Akt threefold (lanes 1&4), which was suppressed by a 4 hr pretreatment with MLK3-agonist IL-1β (compare lanes 2&5 with lanes 1&4). Co-transfection of TRB3 shRNA not only prevented the inhibitory effect of IL-1β on Akt phosphorylation, but also boosted basal Akt activation, for a seven-fold increase in Akt phosphorylation in the presence of insulin (lane 6). Constitutively active Akt suppresses TRB3 expression, and BAX conformational change: Finally, we examined whether overexpression of Akt could compensate for inhibitory effects of TRB3 and rescue cytokine-dependent, TRB3-mediated, BAX conformational change (Fig 6B). Adenoviruses encoding kinase dead (KD) or constitutively active myristoylated (Myr) Akt (rows i and ii), were expressed in Min6 cells, and tracked by coexpressed GFP (pseudocolored blue column 1). For visual clarity of TRB3 and BAX colocalization in yellow, TRB3 fluorescent signal was pseudocolored green (column 2) and BAX-6A7 was visualized using Alexa-fluor 568 (column 3). Stimulated medium again resulted in induction of TRB3 and co-staining with BAX-6A7 (arrowheads, column 4). As depicted in a cartoon representation in column 5, more of the TRB3, BAX-6A7 double positive cells (in yellow) were excluded from the Akt-Myr expressing cells (row ii), compared with Akt-KD (row i) expressors. TRB3 and BAX-6A7 double positive cells were quantified and expressed as a percent of total virus-expressing (GFP+) population (Fig 6C). Taken together and as represented in Fig 6D, our data suggest that cytokine-dependent induction of MLK3 and TRB3 work together to inhibit the protective effects of Akt on mitochondria in the beta cell, and weaken cellular resistance to other cytokine-activated effectors.

**DISCUSSION**

In this study we have identified MLK3 as a pivotal kinase in cytokine-activated beta cell death, using both primary islets and beta cell lines. MLK3 was induced by the complex mix of cytokines secreted by activated splenocytes, as well as by purified IL-1β. Since static incubation of islets with a cocktail of cytokines results in little (44) or late induction (5-10 days) of beta cell death (45), we devised a system that would better represent the milieu of insulitis encountered by islets in vivo. Cell death pathways were examined using an ex vivo system where islets were co-cultured with activated splenocytes. Using this system we found that MLK action was focused on compromising mitochondrial integrity in the early stages (4-16 hours) of beta cell death, by inducing conformational change and mitochondrial translocation of BAX. More importantly, we identified TRB3, a negative regulator of Akt, as a novel and key downstream mediator of MLK3 effects.

Akt can neutralize pro-apoptotic events by multiple mechanisms including inhibiting conformational change and mitochondrial translocation of BAX (15,16). Accordingly, in Min6 cells, in response to cytokines, more than 95% of TRB3 expressing cells were also positive for BAX conformational change, and both were inhibited by the knockdown of either MLK3 or TRB3 (schematic for model represented in Fig 6D). These data highlight a close correlation between TRB3 induction and BAX conformational change, and show that in the beta...
cell, both events are downstream of MLK3. Thus our findings demonstrate that in the pancreatic beta cell, activation of MLK3 not only regulates JNK activation (Fig 1C, and E) and its potential apoptotic effects (reviewed in (46,47)), but as reported here, MLK3 also lowers Akt-dependent resistance to apoptosis via TRB3. Mechanistically, we found that MLK3 binds and stabilizes and raises cellular levels of TRB3 to inhibit the survival kinase Akt.

In the beta cell, MLK3 and TRB3 were required for cytokine-mediated mitochondrial translocation of BAX. Permeabilization of the outer mitochondrial membrane following insertion of conformationally altered BAX oligomer often represents a commitment to cell death (17,18). Akt increases cell survival by deploying multiple mechanisms that converge on preserving mitochondrial integrity and some of these directly engage BAX (48,49). However, other pathways have been shown to contribute toward beta cell apoptosis including events driven by NFKB, and FoxO transcription factors (31,50). A key target for these pathways is iNOS, which increases oxidative stress by inducing reactive oxygen species and nitrosylation of proteins (51). Interestingly, in our model, iNOS induction at 8-12 hours was unresponsive to MLK inhibition (Fig S6), and occurred later than the effects of MLKs on the mitochondria (at 4-8 hours, Fig 3A-D). The focused effect of MLKs on compromising mitochondrial function, with little effect on some of the above events, suggests that additional MAP3Ks may be involved in regulating parallel aspects of the apoptosis program.

Proinflammatory cytokines have been shown to induce pancreatic beta cell death via apoptosis as well as necrosis, and compromise in mitochondrial function has been proposed to be a prominent event in beta cell death (52). Permeabilization of the mitochondrial membrane results in activation of potent proteases and caspases, and secondary activation of endonucleases leading to apoptosis (53). On the other hand, mitochondrial permeabilization has been associated with a decrease in bioenergetic capacity, dysfunction of the electron transport and production of superoxide anions (54). Our finding that MLK3 impacts mitochondria early in the death cascade, is likely to increase the effectiveness of later events, irrespective of the mode of cell death (53,54). We interpret these data to suggest that MLK and TRB3 driven mitochondrial instability may be important to confer “death competence” to the beta cell.

Robust activation of MLK3 in islets of NOD diabetic mice suggests a role for MLK3 in type 1 diabetes. In the beta cell, cytokine-mediated activation of JNK was MLK dependent (Fig C and S5B). However, the ability of MLK3 to induce cellular apoptosis is context-dependent, as evidenced by its ability to regulate cell proliferation via ERK kinases (55,56) and tissue morphogenesis including regulation of neural tube closure during development (57). In the mature beta cell, we find that in addition to JNK, the pseudokinase TRB3 is a novel and critical non-kinase effector of MLK action. Thus, in our model, actions of JNK and TRB3 converge on BAX, and both are regulated by MLKs in the beta cell, raising the profile of MLKs as potential target in treatment of type 1 diabetes.

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FOOTNOTES

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The abbreviations used are:

SICC: Splenocyte Islet Co-Culture
MLK: Mixed lineage kinase
TRB3: tribbles homolog 3
NOD: Non-Obese Diabetic
MOMP: Mitochondrial Outer Membrane Permeabilization
GST: Glutathione S-Transferase
GFP: Green Fluorescence Protein
RFP: Red Fluorescence Protein
Myr: Myristolylated
ANOVA: Analysis of Variance
FIGURE LEGENDS

Fig. 1. MLK3 is activated in leukocyte-infiltrated islets of NOD mice, by IL-1β in Min6 cells and in Splenocyte Islet Co-Culture (SICC). (A) Pancreatic sections from prediabetic (panel i), and diabetic NOD mice (panels ii-iv) were stained for MLK3 (panels i-iii) and phospho-MLK3 (panel iv) as described. Leukocyte infiltration is outlined. Bar=20µm. (B) Western Blotting of extracts (60µg protein) from Min6 cells treated for 20 mins with 10nM TNF-α, 40ng/ml IFN-γ or 20ng/ml IL-1β using anti-MLK3 antibodies and beta-tubulin used as a control. (C) Western blots for time dependent JNK activation from Min6 cells treated with IL-1β (20ng/mL) in the absence (lanes 2-4) or presence of MLK3 inhibitor CEP11004 (lanes 5-7). (D) Schematic of Splenocyte Islet Co-Culture (SICC). (E) Western blots for total MLK3 and pJNK from of SICC islets co-cultured with increasing numbers of splenocytes (4X & 10X for 30 mins) in the absence (lanes 1-3) or presence of CEP11004 (lanes 4 & 5). Total JNK levels serve as control.

Fig. 2. MLK-dependent induction of proapoptotic markers in Splenocyte Islet Co-Culture (SICC). (A) SICC for 24 hrs with unstimulated (row i), and stimulated splenocytes pretreated with DMSO (row ii) or with CEP11004 (row iii), were used to detect MLK3, TUNEL, TRB3 and conformationally altered BAX using monoclonal antibody clone 6A7 as described (columns 1-4 respectively). (B) Quantification of TUNEL (*P<0.001 versus unstimulated media; **P<0.001 versus stimulated media as tested by ANOVA followed by Bonferroni post hoc test) represents means ± SEM of three independent experiments. (C) Pancreatic sections from prediabetic and diabetic NOD mice were immunostained for TRB3 (i-ii) or BAX-6A7 (iii-iv). Bar=20µm.

Fig. 3. Cytokines upregulate TRB3, compromise mitochondrial function and induce apoptosis in an MLK-dependent manner. (A) Colocalization of endogenous TRB3 with conformationally-altered BAX (row i) in Min6 cells treated for 8 hrs with unstimulated (column 1) or stimulated (column 2-4) medium. Arrowheads show colocalization of TRB3 (green) and BAX-6A7 (red), bar=10µm. Schematic representation of TRB3 and BAX-6A7 colocalization is shown in row ii. (B) Confocal microscopy of Cos-7 cells transfected with RFP or BAX-RFP fusion constructs in the presence of CMV control or CMV HA-TRB3. Mitochondria were tracked with deep-red mitotracker dye (pseudocolored green). Bar=1µm. (C and D) Western blotting of 60µg protein from Min6 cells treated with stimulated medium (4 hrs) from SICC (C) or 20ng/ml of IL-1β (D) in the absence (Fig B lane 2, Fig C lanes 2-4) or presence of CEP11004 (Fig B lane 3, Fig C lanes 5-7). (E) Western Blotting (15µg protein) of cytoplasmic (Cyto) and mitochondrial (Mito) fractions for BAX or cytochrome C, from Min6 cells treated for 8 hours with unstimulated (-) or stimulated (+) medium, with or without CEP11004. Tubulin and porin served as controls for cytoplasmic and mitochondrial fractions respectively. (F) J-aggregates in Min6 cells incubated for 16 hrs with unstimulated (panel i) or stimulated medium, with DMSO (panel ii) or CEP11004 (panel iii) followed by JC-1 dye treatment for 15 mins, bar=2µm. Change in mitochondrial potential was assessed by FACS analysis of J-aggregates (panel iv). TUNEL detection (panels v-vi; Bar=40µm) and quantification (panel viii) of apoptotic Min6 cells treated as described for panels i-iii. (*P<0.05 versus unstimulated media; **P<0.05 versus stimulated media, as tested by ANOVA followed by Bonferroni post hoc test).

Fig. 4. Cytokine-induced BAX conformational change, and apoptosis requires MLK3 and TRB3. (A) Min6 cells were cotransfected with shRNA constructs U6-GL3 (row i and ii), U6-MLK3 (row iii) or U6-TRB3 (row iv), with RFP (pseudocolored blue, column 1) to track transfected cells. Induction of endogenous TRB3 (green-column 2) and its colocalization with BAX-6A7 (column 3, pseudocolored red) was detected following a 8 hr treatment with unstimulated (row i) or stimulated (row ii-iv) medium. Column 4 shows merge of RFP (pseudocolored blue) marker to track shRNA uptake, BAX-6A7 and TRB3 signals. Arrowheads show colocalization of TRB3 and BAX-6A7 in the absence of shRNA. Arrows indicate TRB3 and BAX-6A7 in the presence of shRNA control U6-GL3. Bar=10µm. Schematic representation of the overlapping expression of shRNA (blue) and TRB3/BAX-6A7 double positive cells
(yellow) is shown in column 5. (B) TUNEL analysis of Min6 cells co-transfected with RFP reporter and either U6-GL3 (panel i), U6-MLK3 (panel ii), MLK3-KD (panel iii), or U6-TRB3 (panel iv), were treated with stimulated medium for 24 hr. Arrows: TUNEL, shRNA/RFP double positive. Bar=1µm. (C) Quantification (means±SD of three independent experiments) of shRNA positive cells co-expressing BAX-6A7 and TRB3 (*P<0.001 versus unstimulated media; **P<0.001 versus U6-GL3, as tested by ANOVA followed by Bonferroni post hoc test). (D) TUNEL quantification of RFP positive cells (Mean±SD of three experiments, *P<0.001 versus U6-GL3, as tested by ANOVA followed by Bonferroni post hoc test).

Fig. 5. MLK3 binds and stabilizes TRB3 protein. (A) In vitro protein-protein interaction using bacterially expressed GST-MLK3-WT (lanes 1 & 3) and GST-MLK3-KD (lanes 2 & 4) to pulldown in vitro transcribed and translated (IVT) HA-TRB3 (lanes 3-5), or reticulocyte lysate programmed with empty vector (lanes 1 & 2), followed by western immunoblotting (IB) for TRB3 (upper panel). Total input of MLK3 as Coomassie stained proteins (middle panel) and their phosphorylation status (lower panel) are shown. (B) TRB3 and MLK3 interactions were assessed from lysates of cells co-transfected with HA-TRB3-WT (lanes 2, 3 and 5) and either GST-MLK3-WT (lanes 1-3) or GST-MLK3-KD (lanes 4-5) by GST-pulldown of cellular extracts (PD) followed by western blotting. Blots of GST-pulldowns (panels i and iii) or 7.5% input (panels ii, iv and v) using antibodies against pMLK3 (panel i) TRB3 (panel iii and iv) total MLK3 (panel ii) and co-transfected control Flag-ß-tubulin (panel v) are shown. Lane 3 shows effect of 60’ treatment with MLK inhibitor CEP11004. (D) MLK3 interacts with the N-terminus of TRB3. GST pulldowns (PD) from cells co-transfected with GST-MLK3-WT (lanes1-4) and HA-TRB3-WT (lane 1) or TRB3 mutants ΔN94, ΔN201, ΔC310 (lanes 2-4 respectively) followed by western blotting using anti-MLK3 (top panel) and anti-HA (middle panel) antibodies. Expression levels of TRB3 constructs were assessed using 7.5% input of total cellular lysate using anti-HA antibodies (bottom panel). (D) Schematic of TRB3 deletion mutants. (E) Time-dependent cycloheximide chase of HA-TRB3-WT (lanes 1-4) and HA-TRB3-Δ94 (lanes 5-8) immunoprecipitated using anti-HA beads from cells co-expressing GST-MLK3-WT followed by western blotting using anti-MLK3 and anti-HA antibodies. Cotransfected Flag-ß-tubulin was used as a stable control. (F) Densitometric quantification of time-dependent TRB3 loss to determine half-life (T1/2) of TRB3-WT (closed circles T1/2= 3hrs) or TRB3-Δ94 (open circles T1/2= 45mins) in the presence of catalytically active MLK3.

Fig. 6. Cytokine-induced expression of TRB3 inhibits Akt activity and results in BAX conformational change. (A) TRB3 knockdown rescues cytokine dependent inhibition of Akt. Myc immunoprecipitates from cells transfected with Myc-Akt, and shRNA constructs U6-GL3 (lane 1-2 & 4-5) or U6-TRB3 (lanes 3 & 6) were analyzed for phospho-S473-Akt and total Myc-Akt using western blotting. Prior to IP, cells were treated with vehicle or 20ng/ml IL-1β for 4 hours (lanes 2-3 & 5-6) followed by 20 min stimulus using 20nM insulin (lanes 4-6). Densitometric quantification of fold difference in Akt phosphorylation is included below each lane. (B) Constitutive Akt activity rescues cytokine-induced TRB3 expression and BAX conformational change. Min6 cells transduced with adenovirus expressing GFP-Akt-KD (kinase-dead) (row i) or GFP-Akt-Myr (myristoylated) (row ii) were treated with stimulated medium for 4 hrs and immunostained for induction of endogenous TRB3 (column 2; pseudocolored green for clarity) and conformationally altered BAX (6A7) (column 3). GFP expression (column 1; pseudocolored blue for clarity) marked the transduced cells. Column 4 shows TRB3/BAX-6A7/GFP merge. Arrowheads show colocalization of TRB3 with BAX-6A7; arrows show TRB3 and BAX-6A7 colocalization with Akt-KD. Bar=10µm. Schematic representation of the overlapping expression of transduced cells (blue) and TRB3/BAX-6A7 double positive cells (yellow) is shown in column 5. (C) Quantification of GFP+ cells co-expressing BAX-6A7 and TRB3 (means±SD of three independent experiments, *P<0.001 versus unstimulated media; **P<0.001 versus Akt-KD, as tested by ANOVA followed by Bonferroni post hoc test). (D) Schematic of MLK and TRB3 in cytokine-activated beta cell apoptosis. Solid and dotted lines indicate direct and indirect effects respectively.
SUPPLEMENTARY FIGURE LEGENDS

**Supp. Table 1.** Cytokine secretion in SICC. Average cytokine secretion by splenocytes isolated from diabetic NODmice in response to co-stimulation with anti-CD3 and anti-CD antibodies. Cytokine analysis was conducted as instructed by the manufacturer using the Beadlyte Mouse Multi-Cytokine Detection System (Upstate, Charlottesville, VA) and the Luminex 100 LabMAP System, with cytokine concentrations interpolated against the linear range on the standard curves (Softmax Pro, Molecular Devices, Sunnyvale, CA).

**Fig. S1.** MLKs are activated by IL-1β in human islets. Western blots of human islet extracts (60 µg protein) treated for 20 mins with IL-1β using anti-MLK1-3 and pMLK3 antibodies. Fold induction by IL-1β (n=5) was assessed using densitometry and representative blots are shown. PDX1 was used as a control.

**Fig. S2.** Minimal induction of MLK3, TRB3 and BAX mRNA in response to cytokines. Bar graphs showing MLK3, TRB3 mRNA in response to IL-1β (left) or MLK3, TRB3 and BAX mRNA in response to stimulated media (right). Relative quantification (ΔΔCt) of MLK3, TRB3 and Bax mRNA following treatment of Min6 cells at the indicated timepoints. Real time PCR analysis was performed using SYBR Green mastermix (Applied Biosystems, Foster City, CA), Ct values were generated using ABI Prism 7700HT (Applied Biosystems, Foster City, CA) for MLK3, TRB3 and Bax message (Forward primers (5’-3’) ACCTATGCGCTGTATGGGAG, CAGCAACTGTGAGAGGACGA, TGCAGAGGGATGATGCTGAC; Reverse (5’-3’) GCCTCATCTGGGTGTTTTT, CGGGAGCTGATCTCTGG, GGAGGAATCCATGTCCAG respectively) and were normalized by Sequence Detection Systems software (SDS v2.2.1, Applied Biosystems, Foster City, CA) to GAPDH (Forward 5’ ACCCAGAAGACTGTGGATGG 3’; Reverse 5’ CACATTGGGGGTAGGAACAC 3’). Message level was expressed as fold induction over that observed in untreated cells (for IL-1β) or cells treated with unstimulated media (for stimulated media).

**Fig. S3.** Response of representative members of JNK activating MAP3Ks to CEP11004. (A) GST pulldown assay performed from extracts of Cos-7 cells expressing GST-JNK and either pcDNA3 (lane 1) TPL2 (lanes 2-3), MEKK1 (lanes 4-5), ASK1 (lanes 6-7), TAK1 (lanes 8-9) or MLK3 kinases (lanes 10-11). Transfected cells were cultured in the absence or presence of CEP11004 for 2 hours, prior to harvesting and probed for JNK activation using anti-p-JNK antibody. The blot was stripped and probed for total JNK.

**Fig. S4.** MLK-dependent induction of caspase 6 cleavage in Splenocyte Islet Co-Culture (SICC) and Min6 cells. (A) SICC for 24 hrs (upper panel) with unstimulated (row i), and stimulated splenocytes pretreated with DMSO (row ii) or with CEP11004 (row iii). Using ScanScope Deconvolution Software (Aperio Technologies, Vista, CA), the deconvolved image (middle panel) is generated by setting appropriate threshold levels for positive and negative signals. Threshold levels are the same for all treatments and remain above the positive or negative pixel intensity (see algorithm output in the quantification panel for each treatment below each image). Direct comparison is possible since the entire timecourse of treated islets was arrayed and stained on the same slide. (B) The percent of positive and negative pixels are shown in the lower panel and represented in graphical format (*P<0.0001 versus unstimulated media; **P<0.0001 versus stimulated media; results show means ± SEM of three independent experiments). (C) Cleaved caspase-6 western blot of 60 µg protein from Min6 cells cultured in stimulated medium in the presence (lanes 1-3) and absence of CEP11004 (lanes 4-6).

**Fig. S5.** (A) Knockdown of endogenous MLK3 in cultured βTC-3 cells (used here for high level of transfection). U6 control, U6-GL3 or U6-MLK3 (lanes 2, 3 and 4) were co-transfected with RFP reporter, followed by western blot for MLK3 expression (top panel) in FACS sorted RFP positive cells. JNK was used as control (bottom panel). (B) MLK-dependent induction of JNK in Min6 cells. GST-JNK pulldown

13
probed with anti-pJNK Ab from untreated (NT) or IL-1β treated cells transfected with U6-GL3 or U6-MLK3. Knockdown of MLK3 attenuates IL-1β dependent JNK activation in Min6 cells.

Fig. S6. MLK-independent induction of iNOS in Min6 cells. Time dependent activation of iNOS in Min6 cells in response to conditioned media from stimulated splenocytes (Stim). CEP11004 pretreatment failed to attenuate iNOS activation (Stim + CEP).

Fig. S7. Specificity of MLK3, pMLK3 and TRB3 antibodies. Cells were transfected with either GST-MLK3 WT (A & B, lower panel), GST-MLK3 KD (B, upper panel), HA-TRB3 WT (C, upper panel), or HA-TRB3 ΔN 201 (C, lower panel) and fixed in Z-fix (Anatech, Battle Creek, MI). Double labeling enabled the specificity of the MLK3 and TRB3 antibodies to be compared to the localization of the GST or HA tag. Primary antibodies were revealed with species-specific fluorescent probes and the nuclei counterstained with DAPI. (A) Goat anti-GST tag (Bethyl Laboratories, Montgomery, TX; lane 1) and rabbit anti-MLK3 (0.8µg/ml affinity purified rabbit IgG; A Rana Loyola University, IL; lane 2) are 100% co-localized in the merge images (lane 3). (B) Goat anti-GST tag (Bethyl Laboratories, Montgomery, TX; lane 1) and rabbit anti-phospho MLK3 T277/S281 (1:100; Cell Signaling, Beverly,CA; lane 2) recognizing auto-phosphorylated MLK3 are 100% co-localized only in the cells expressing GST-MLK3 WT (lower panel, lane 3). (C) Mouse anti-HA (Cell Signaling, Beverly,CA; lane 1) and rabbit anti-TRB3 (1:100 rabbit antiserum; Gift of Marc Montminy, Salk Institute; lane 2) only co-localize when the full length TRB3 protein (top panels) is expressed (merged images, lane 3). The TRB3 antibody was generated against the N terminus of the protein.
Figure 1

A. MLK3 in NOD-PreDiab, NOD-Uninfiltrated, NOD-Infiltrated, and NOD-Infiltrated conditions.

B. Western blot analysis of MLK3 and β-Tubulin in different conditions.

C. Time-course of cytokine treatment with IL-1β and Western blot analysis of p-JNK and JNK.

D. Schematic diagram of the experimental setup involving NOD prediabetic and diabetic conditions.

E. Western blot analysis of MLK3, pJNK, and JNK under unstimulated and stimulated conditions.

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Figure 5
Figure 6

A

|          | Control | Insulin |
|----------|---------|---------|
| IL-1β    | -       | +       |
| U6 TRB3  | -       | +       |
| U6 GL3   | +       | -       |
| p473Akt  | -       | +       |
| Akt      | -       | +       |

Fold Diff

1 2 3 4 5 6

78kd 50kd 78kd 50kd

B

|          | AKT-KD | TRB3 | 6A7 | Merge | Schematic |
|----------|--------|------|-----|-------|-----------|
| Stimulated |        |      |     |       |           |
| AKT-KD   |        |      |     |       |           |

C

% 6A7 and TRB3 co-expression

Medium Unstim Stim Stim Stim
Virus - - KD Myr

D

Cytokines

MLK3 MKK7 JNK

Akt

TRB3

Bax translocation

mitochondria

Other pathways

β-cell death
Mixed-Lineage kinase-3 stabilizes and functionally cooperates with tribbles 3 to compromise mitochondrial integrity in cytokine-induced death of pancreatic beta cells
Rohan K. Humphrey, Christina J. Newcomb, Shu-Mei Yu, Ergeng Hao, Doris Yu, Stan Krajewski, Keyong Du and Ulupi S. Jhala

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