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HUMAN TRIBBLES-1 CONTROLS PROLIFERATION AND CHEMOTAXIS OF SMOOTH MUSCLE CELLS VIA MAPK SIGNALLING PATHWAYS

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Running title: Trb-1 regulates smooth muscle cell function

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Migration and proliferation of smooth muscle cells are key to a number of physiological and pathological processes, including wound healing and the narrowing of the vessel wall. Previous work has shown links between inflammatory stimuli and vascular smooth muscle cell proliferation and migration through mitogen activated protein kinase (MAPK) activation, though the molecular mechanisms of this process are poorly understood.

Here we report that tribbles-1, a recently described modulator of MAPK activation controls vascular smooth muscle cell proliferation and chemotaxis via the Jun Kinase pathway. Our findings demonstrate that this regulation takes place via direct interactions between tribbles-1 and MKK4/SEK1, a Jun activator kinase. The activity of this kinase is dependent on tribbles-1 levels, whilst the activation and the expression of MKK4/SEK1 is not. In addition, tribbles-1 expression is elevated in human atherosclerotic arteries compared to non-atherosclerotic controls, suggesting that this protein may pay a role in disease in vivo. In summary, the data presented here suggest an important regulatory role for trb-1 in vascular smooth muscle cell biology.

Injury to the arterial wall initiates a series of changes in specialised molecular and cellular events that not only contribute to wound healing but to the pathogenesis of atherosclerosis, the presentation of acute coronary syndromes (ACS) and the complications of percutaneous coronary intervention (PCI). An important initiating event appears to be endothelial cell dysfunction or cell death caused by local abnormalities. These events result in the production and release of a number of inflammatory cytokines and chemokines. In pathogenic conditions, elevated levels of inflammatory mediators can lead to migration and proliferation of vascular smooth muscle cells (VSMC) to form a neointima. These cellular responses are mediated via the co-ordinated action of various second messenger pathways, including activation of Mitogen Activated Protein Kinase (MAPK) cascades and IκB kinases. Activation of these systems has been reported in several pathological conditions of vessel walls (1-3). MAPKs are involved in the regulation of development, cell activation, proliferation and vascular contraction (4-7). Further, they are central in regulating VSMC activation. It has been demonstrated that activation of MAPK cascades occurs in response to a wide range of stimuli, including pro-inflammatory cytokines, growth factors, mechanical stimuli (stress) (8) and integrin-dependent cell/matrix interactions (9, 10). MAPKs are classified into at least three distinct groups (JNK, p38 and ERK kinases) and can be activated via a variety of upstream kinases, MAPKKs. In VSMC, Jun kinases (JNK) and p38 MAPKs have been implicated in responses primarily to stress (heat, hypoxia, chemical, oxidative, etc.) and pro-inflammatory cytokines, extracellular signal regulated protein kinases (ERK) primarily respond to mitogenic stimuli such as growth factors (PDGF) (11), oxidised LDL (12) or Ang II (reviewed in (13)). However, in most cases a given stimulus will activate more than one group of MAPKs. The specific contribution of each MAPK pathway to a physiological response varies from cell type to cell type. In some cases, MAPK pathways can co-operate, but they can antagonise in others (14, 15).

However, the mechanisms which are responsible for these differences in MAPK responses in VSMC are largely unknown.

We have recently reported the identification (16, 17) and characterisation of a novel protein family, human tribbles (trb) as regulators of MAPKK activity (16, 18). We have shown that trb-1 and trb-3 are able to bind to various MAPKKs and that their concentration regulates preferential activation of the different MAPK pathways, presumably leading to different cellular responses (18). Drosophila and Xenopus Tribbles have been shown to regulate cell cycle progression during embryonic development (19-22). Murine trb-3 has been demonstrated to inhibit insulin-dependent activation of Akt and was suggested to play a role in the development of diabetes in a mouse model (23, 24). However, recent conflicting data suggest that this proposed role may require further clarification (25). Further, the importance of human tribbles proteins in cell physiology has not been evaluated. Here we report that trb-1 is selectively overexpressed in chronically inflamed human atherosclerotic arteries and that it regulates vascular smooth muscle cell proliferation and chemotaxis via the JNK pathway. These observations define trb-1 as a novel, central regulator of VSMC function.

EXPERIMENTAL PROCEDURES

All the experiments described in this study were performed multiple times (N>2) and representative datasets are shown.

Ethics:

The human samples were obtained under the ethical approval granted by the North Sheffield Research Ethics Committee. This study conforms to the principles outlined in the Declaration of Helsinki.

Plasmids, siRNA

Trb-1 overexpression plasmid has been described before (18). siRNA SmartPool against human trb-1 was purchased from Dharmacon and used according to the manufacturer's recommendation. Plasmids for yellow fluorescent protein (YFP)-based protein fragment complementation assay (PCA) (42, 43) were a kind gift of Prof. S. Michnik and have been described before (26).

Cell culture and the stimulation with LPS

VSMC were purchased from Cascade Biologics and maintained in Medium 231 with smooth muscle growth supplement (all from Cascade Biologics). VSMC were plated in 6 well plates at an initial density of 0.5×10^5 cells/well in 2 ml of complete growth media. 24 hours post transfection cells were stimulated by 100 ng/ml LPS, as stated in the figures. HeLa cells (ATCC) were maintained in DMEM medium (Invitrogen), containing 10% Foetal Bovine Serum (Biowhittaker), 5mM of sodium pyruvate and 100 µg/ml of penicillin and streptomycin (Sigma).

Artery biopsies

Coronary arteries from patients with a diagnosis of ischemic heart disease (IHD, n=8) and dilated cardiomyopathy (DCM, n=6) were harvested from transplantation recipients at the time of surgery. Patients diagnosed as having DCM were free of CAD before transplantation, as assessed by coronary angiography and histological analysis by a cardiac pathologist (SKS) using the American Heart Association (AHA) classification system (27-29). All patients were men and white in origin. Mean ages were 55.75 ±5.11 for IHD and 48.5±10.23 for the DCM group. Mean AHA histological grades of disease were 6.42 for the IHD and 3.16 for the DCM group, respectively.

RNA isolation and Quantitative Real-time PCR analysis

Total RNA was extracted from human artery biopsies and VSMC using RNeasy kit (Qiagen) according to the manufacturer's protocol. 2µg RNA was reverse transcribed into first strand cDNA and used immediately for qRT-PCR. Gene expression was analyzed by qRT-PCR using ABI prism 7900 (Applied Biosystems). Probes for human trbs, IL-1 β, IL-RA, TNF α and GAPDH were synthesized by Sigma Genosys with FAM at the 5'-end and TAMRA at the 3'-end. The sequences of all primers and probes used are listed below.

Target		Sequence(5'-3')
htrb-1	Forw	CCCCAAAGCCAGGTGCCT

	ard	
	Reverse	TACCCGGGTTCCAAGACG
	Probe	FAM-CAGCCTCTTGAGACGGGA-TAMRA
htrb-2	Forward	CATACACAGGTCTACCCCC
	Reverse	TCCGCGGACCTTATAGAC
	Probe	FAM-CTTCGAAATCCTGGGTTTT-TAMRA
htrb-3	Forward	CTGCCCGCTGTCTGGTTC
	Reverse	GGGCATCGGGTCCTGTCG
	Probe	FAM-TGCCTCCTTCGTCGGGAG-TAMRA
IL-1 β	Forward	ACAGATGAAGTGCTCCTTCCA
	Reverse	GTCGGAGATTCGTAGCTGGAT
	Probe	FAM-CTCTGCCCTCTGGATGGCGG-TAMRA
IL-RA	Forward	GAAGATGTGCCTGTCCTG
	Reverse	CGCTCAGGTCAGTGATGT
	Probe	FAM-TGGTGATGAGACCAGACT-TAMRA
GAPDH	Forward	GCCTTCCGTGTCCCCACT
	Reverse	TGAGGGGGCCCTCCGACG
	Probe	FAM-CCTGCTTCACCACCTTCTT-TAMRA

To quantify transcripts for genes of interest, we used the GAPDH transcript as an internal control, and each sample was normalized with respect to GAPDH transcript content. Standard curves for the 9 genes and GAPDH mRNAs were generated using serially diluted solution of plasmids incorporating each gene as a PCR template. All assays were performed in duplicate and the average values were used for analysis.

Transfections:
Transfections were performed using Nucleofector (Amaxa) using program U-25 and Nucleofector solution for VSMC (Amaxa). For most experiments, 1.0×10^6 cells were used per nucleofection. The rate of transfection was >40 as judged by EGFP expression, measured by fluorescent microscopy (data not shown). Polyfect (Qiagen) was used for transfection of HeLa cells according to the manufacturer's instructions.

Proliferation assay:
Transfected VSMC were placed on 96 well culture plates and further cultured for 24 hours. Cells were then treated with [^{3}H] thymidine (1 μCi/well) and /or MAPK inhibitors for 6, 24, 30hours. ERK MAPK inhibitor (PD98059), p38 MAPK inhibitor (SB203580) and JNK MAPK inhibitor (SP600125) were purchased from Calbiochem and used at 20μM for ERK and JNK MAPK inhibitors and 0.2μM for p38 MAPK inhibitor. After treatment, [^{3}H] thymidine incorporation was measured by liquid scintillation counting.

***In Vitro* wound healing assay, Time Lapse Video-Microscopy (TLVM):**
Cells were transfected as described above, seeded into 35mm culture dishes and incubated overnight. Confluent cell monolayers were wounded by removing a strip of cells from the plate surface with a standard 1ml pipette tip. Wounded monolayers were washed with PBS to remove non-adherent cells and replaced with fresh complete growth medium. TLVM (30, 31) was used to film migration at the wound edges for 24 hours (1 frame every 2.5minutes).
The number of cells crossing a line marking the wound edges was counted over the 24 hour period. (Fig. 2D)
15 randomly picked cells from the wound edges were tracked over a 1 hour period. The on-screen distance travelled was measured to calculate migration speed (mm/h) irrespective of direction. (Fig. 2E)

Western blotting:
Anti-phospho-MAPK and anti-MAPK antibodies were purchased from Cell Signalling Technology and were used according to the manufacturer's recommendations. Anti-β-actin antibody was from Dako. Between probing for the various proteins, membranes were stripped by Re-Blot Plus Mild solution (Chemicon). Membranes were developed with SuperSignal West Pico Chemiluminescent Substrate and signals were quantified by Chemigenius gel-documentation system (Syngene). pMAPK and MAPK signals were expressed as a ratio to β-actin levels within the same sample.

Immunostainning
Serial 4μm thick paraffin-embedded human artery sections were stained for Trb-1 using standard techniques. Trb-1 primary antibody (Millipore-Upstate) was applied overnight at 4 ^{o}C. Sections were then exposed to goat biotinylated anti-rabbit secondary antibody (Vector laboratories) for 30 minutes at room temperature. Vectastain ABC reagent (Vector Laboratories) was added and incubated at room temperature for 30 min. Slides were then immersed in 0.25 mg/ml 3,3'diaminobenzidine, activated with hydrogen peroxide and counterstained with Carazzi's hematoxylin. Primary antibody was omitted from the negative controls.

RESULTS

Tribbles expression is regulated in response to an inflammatory stimulus in hASMC
Smooth Muscle Cell proliferation is a key event in the healing response to injury, a process which is initiated by inflammatory stimuli (32). To identify whether tribbles expression is regulated in vascular cells under inflammatory conditions, we stimulated human aortic smooth muscle cells (hASMC, Fig. 1A) and human umbilical vein endothelial cells (HUVEC, not shown) with LPS and measured tribbles mRNA expression levels by using qRT-PCR. IL-1β expression was measured as positive controls in the same samples (Fig. 1B). We found that trb-1 was selectively and transiently up-regulated by LPS treatment in hASMC (Fig. 1A) but not in HUVEC (not shown). Previous reports (33) and our unpublished observations suggest that tribbles proteins may be unstable and expressed at low levels, therefore, mRNA levels are likely to correlate well with protein expression. We have, therefore, investigated the biological relevance of altered trb-1 levels on hASMC under inflammatory conditions.
To assess the role of trb-1 in LPS stimulated hASMC, we transiently transfected these cells with a trb-1 expression plasmid or a pool of anti-trb-1 siRNA oligonucleotides and measured proliferation and migration in response to PDGF. However, first we evaluated the potential impact of culture conditions on trb-1 expression (Fig. 1C) and the specificity of the siRNA pool (Fig. 1D) used for targeting trb-1 mRNA. We have found that whilst trb-1 siRNA had the desired activity both under standard culture conditions (10% FCS) and when cells were serum starved post-transfection (0.5%), the expression levels of this gene and, indeed, the other tribbles as well (not shown) were significantly affected by the serum concentration. This is in line with previous work, demonstrating that expression of members of mammalian trb family is modulated through metabolic signals (34, 35). Therefore, we have used 10% FCS in all our experiments in order to minimise inter-experimental variations. In addition, the siRNA pools showed high specificity towards trb-1 and did not alter significantly trb-2 and -3 expression (Fig. 1D). In line with these results, siTrb-1 treatment resulted in a substantial decrease in trb-1 protein levels (Fig. 1E)

Tribbles-1 regulate specific hASMC cellular functions
To investigate the role of trb-1 in hASMC function, we raised or suppressed trb-1 levels by transient transfection of hASMC cells with trb-1 expression plasmid or siRNA and measured proliferation by ^{3}H-thymidine incorporation (Fig. 2A-B). We observed a modest anti-proliferative effect at 48 hrs post-transfection (24 hrs time point in the proliferation assay) when trb-1 was overexpressed. In contrast, depletion of trb-1 results in a significant increase in proliferation rate at the same time-point (Fig. 2B). However, this assay measures the net effect of cell death and proliferation. In order to confirm that the observed increase in ^{3}H-thymidine incorporation is due to an increased mitosis rate, we performed time lapse video imaging on control and si-trb-1 treated cells and calculated the rate of mitosis (Fig. 2C). The data

obtained by both methods was in agreement. In addition, there was not difference in the observed rate of apoptosis between treatment, further supporting a specific tribbles effect on proliferation. Using the same time lapse assay, we also measured VSMC migration in a "wound healing" assay. (Fig. 2D, E). These data show that neither the number of cells migrating into the wound (Fig. 2D) nor their migration speed (Fig. 2E) were affected upon depletion of trb-1.

As trb-1 acts via modulating MAPK activation and this signalling system is known to govern VSMC proliferation, we used selective MAPK inhibitors to block individual pathways and investigated their involvement in the trb-1 regulated proliferation (Fig. 2F). These data demonstrate that blocking of JNK but not p38 pathways suppresses VSMC proliferation. In line with previous reports, inhibiting ERK activation also caused a significant inhibition of proliferation (36).

To further investigate the impact of trb-1 levels in hASMC function, chemotaxic migration of these cells was measured in a transwell migration assay, in response to PDGF (Fig. 2G). Depletion of trb-1 led to an increase in transmigrated cells. Further, inhibition of the JNK pathway abrogated the effect of si-trb-1 treatment, suggesting that trb-1 may be a negative regulator of hASMC chemotaxis via inhibitory activity of the JNK pathway.

Activation of Vascular Smooth Muscle Cells by inflammatory signals leads to the production of a number of cytokines, including TNFα and TGFβ through MAPK mediated signalling events (37-41). We, therefore, investigated whether depletion of trb-1 had a modulator role in the production of these inflammatory cytokines, in VSMC. Our data show no difference in the dynamics or the amplitude of cytokine expression at the TNFα mRNA (Fig. 2H) and TGFβ protein levels (Fig. 2I), implying that trb-1 may be a specific regulator of VSMC proliferation and migration.

Overexpressed Trb-1 blocks AP-1 activation and Trb-1 depletion leads to constitutive JNK activation

In order to gain mechanistic insight into the regulation of VSMC proliferation via the JNK/AP-1 pathways and trb-1, VSMC was transiently transfected with an AP-1 reporter plasmid, activated by overexpressed MEKK1 in the presence and absence of overexpressed trb-1 (Fig. 3A). The results demonstrate that activation of AP-1 can be blocked by overexpressed trb-1 in VSMC, in line with our previous report in HeLa cells (18).

Next, the activation of the various MAPKs were assessed in control and trb-1 depleted cells. Phosphorylated MAPK (pMAPK, the activated form) and total MAPK levels were investigated in response to LPS treatment, by western blotting. pMAPK (Fig. 3B) and total MAPK (Fig. 3C) levels were normalised to β-actin and expressed as relative units. In agreement with our previous report (18), alterations in trb-1 levels had a differential impact on the various MAPK pathways. However, these effects were different in VSMCs, compared to those observed in HeLa cells (18). In VSMC, the amount of phospho-p38 protein but not the dynamics of activation was influenced by altered trb-1 expression. In contrast, while ERK and JNK pathways were also sensitive to altered trb-1 expression, these were no longer up-regulated by LPS stimulation. Indeed, modulation of trb-1 expression caused phosphorylation of both of MAPKs to decrease, once stimulated. In addition, depletion of trb-1 mRNA by the siRNA constructs led to an increase in phosphorylation of JNK in the absence of any stimulus (Fig. 3B, middle graph, zero time point), suggesting that normal trb-1 levels inhibit the activation of this pathway in the non-stimulated state. However, no significant alterations in total MAPK levels were observed in cells, where trb-1 levels were perturbed (Fig. 3C). These observations are compatible with the hypothesis that MAPK scaffold levels are key determinants of pathway activation (42) and that tribbles may have a scaffold-like function as proposed previously (18). According to these models, both up and downregulation of scaffold levels may lead to impaired activation of signalling systems. We have seen such effects on the JNK and ERK pathways in VSMC, similarly to our previous studies on HeLa cells (18).

Trb-1/MKK4 interaction is key to the regulation of hASMC proliferation

We have previously shown in biochemical assays that tribbles proteins interact with MAPKKs and regulate their activity (18). However, since many

aspects of tribbles action appear to be cell type specific (for recent reviews see (43, 44)), we set out to investigate details of trb/MAPKK interactions in hASMC. Our data point to the JNK pathway as a key system in regulating proliferation and chemotaxis in these cells, in a trb-1 dependent manner. Therefore, we characterised the involvement of MKK4/SEK-1 and MKK7, the two known MAPKKs, which lead to activation of JNK. Our results show that MKK4 but not MKK7 is expressed in these cells and that this expression pattern is not influenced by sitrb-1 treatment (Figure 4A). We also found that the phosphorylation of MKK4 was not affected by depletion of trb-1 (Figure 4B), suggesting that trb-1 directly interferes with MKK4 activity, rather than with activation and/or expression. To confirm the direct role of MKK4 in hASMC proliferation, we performed a [^{3}H] thymidine incorporation assay in siMKK4 treated cells (Figure 4C), as before. In line with our above model, the data demonstrate a positive role for MKK4, since depletion of this protein led to a decrease in proliferation rate.

To confirm the physical interaction between trb-1 and MKK4 in live hASMC, we used a yellow fluorescent protein (YFP)-based protein fragment complementation assay (PCA) (45, 46) and co-immunoprecipitation. The Venus variant of YFP was used in this assay, since it provides a higher signal than EYFP. MKK4 and Trb-1 were fused to the N-terminal fragment of Venus YFP (V1) or to the C-terminal portion of Venus YFP (V2), respectively. The two expression constructs were co-transfected and the YFP signal was visualised by fluorescent microscopy (Figure 4D). These data demonstrate that the MKK4/trb-1 complex is located predominantly in the nucleus of hASMC. In line with this finding, a co-immunoprecipitation experiment further confirmed the interaction between MKK4 and trb-1 (Fig. 4E).

We have shown recently that the N-terminal region of trb-1 governs the intracellular localisation of the protein (17). We wondered, therefore, whether the same domain influenced the location of the MKK4/trb-1 complex. Truncated trb-1 forms, lacking the N-terminal, the C-terminal or both domains (Figure 4F) were expressed as V2 fusion proteins (Figure 4E). The results show that the nuclear localisation of the complex is critically dependent on the presence of the N-terminal trb-1 domain. Mutants lacking this domain still showed an interaction with MKK4, but the signal was no longer preferentially nuclear. Further, these experiments demonstrate that the central, kinase-like domain of trb-1 is sufficient for interaction with MKK4. This is in line with our previous report, where we showed that expression of this domain was sufficient to inhibit of AP-1 activation via trb-1 (17).

In order to confirm specificity of the observed interaction, several control experiments were performed. Co-expression of an increasing amount of "unlabelled" trb-1 led to a dose-dependent elimination of the YFP signal as detected by FACS (Figure 4G) or by fluorescent microscopy (Figure 4H). In addition, neither MKK4-Venus nor trb-1-Venus fusion proteins interacted with their zip-Venus counterparts (these were used as positive control constructs in the system) (Figure 4G), which further supports the specific nature of this interaction.

Tribbles-1 expression in atherosclerotic arteries

Proliferation of vascular smooth muscle cells is one of the hallmarks of the development of chronic diseases of the vessel wall. In order to evaluate the potential role of tribbles in human disease, we studied segments of whole artery wall taken from the explanted hearts of patients undergoing cardiac transplantation for ischaemic heart disease (IHD) and characterised tribbles expression. Coronary arteries from patients with non-ischaemic dilated cardiomyopathy (DCM) were used as controls. In order to quantify potential differences in trb expression levels between the two groups, mRNA levels of known pro and anti-inflammatory cytokines (IL-1β and IL-1ra) and tribbles 1-3 were quantified by using qRT-PCR (Fig. 5). As in our previous work (47), we detected a "pro-inflammatory phenotype" in the atherosclerotic group. Expression of trb-1 but not of trb-2 was significantly raised in the IHD group (Fig. 5A), whilst trb-3 expression was not detected in these samples (not shown).

In order to visualise trb-1 expression *in vivo*, sections of a human coronary artery were stained by anti-trb1 antibody (Fig. 5B). The majority of trb-1 specific staining was detected in the intimal and medial areas. Of note, nuclear and/or cytoplasmic trb-1 staining was observed in medial VSMC.

DISCUSSION

In this paper, we have evaluated the importance of tribbles proteins, particularly trb-1 in the cellular responses of vascular smooth muscle cells to inflammatory stimuli. Taken together, our experiments demonstrate that trb-1 is found in VSMC *in vivo* and that expression levels are key in modulating the extent of VSMC proliferation and chemotaxis. In contrast, VSMC migration speed in a "wound healing" assay and the production of cytokines are not affected by altered trb-1 levels, suggesting a specific physiological role for this protein. Whilst a body of literature supports the involvement of ERK and JNK MAPK pathways in VSMC chemotaxis, the role of these second messenger systems in wound healing assays (in the absence of chemokines) is much less understood. Our data implies that the two processes might be differentially regulated. However, further detailed studies will be needed to clarify this.

Blockade of specific MAPK pathways by pharmacological inhibitors pointed to the JNK pathway as a major regulator of VSMC proliferation. In line with the model where trb-1 controls JNK activation, we found that si-trb-1 treatment of VSMC led to the spontaneous activation of JNK (Figure 3B). Therefore, we have investigated the detail of the interaction between JNK activating kinases and trb-1. Of the MAPK kinases, which are known to activate JNK, MKK4 but not MKK7 was found to be expressed in VSMC. Using a recently developed technique (PCA), we were able to visualise the interaction between trb-1 and MKK4 in live VSMC cells. To our knowledge, this is the first use of this powerful technique in studies of primary vascular cells. A number of controls demonstrate the specificity of the observed interaction. Further, we were able to show that the intracellular localisation of the trb-1/MKK4 complex is dependent on the N-terminal domain of trb-1. This is in line with our previous findings where we demonstrated that the N-terminal domain of trb-1 is essential for nuclear localisation. However, clarification of the functional relevance of this observation requires further studies. The use of truncated trb-1 proteins in PCA demonstrates that the kinase-like domain of trb-1 is essential for it's ability to interact with MAPKKs. Since this domain is similar to that of the MAPKs (MAPKK substrates), a plausible hypothesis for the molecular mechanism of trb action is that tribbles may compete for the binding site with the MAPKs, thus regulating their activation. This model may explain why evolution preserved a catalytically inactive kinase domain from unicellular organisms to mammals (43, 44).

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FOOTNOTES

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FIGURE LEGENDS

Figure 1: trb-1 expression in cultured human Aortic Smooth Muscle Cells. hASMC were stimulated by LPS for the various time points as indicated, total RNA was prepared and qRT-PCR was performed to detect changes in **(A)** tribbles 1-3 mRNA expression levels. **(B)** IL-1β levels were also measured as positive controls in the same samples. **(C)** The impact of culture conditions on Trb-1 expression was assessed in control and si-trb-1 transfected hASMC cells by qRT-PCR. **(D)** The specificity of si-trb-1 knockdown was evaluated by qRT-PCR, comparing expression levels of trb-1, -2 and -3 in si-trb-1 transfected cells. The values were normalised to the expression of tribbles in cells transfected with control siRNA. (E) The efficiency of si-trb-1 treatment was assessed by western blot. Cells were transfected with control or trb-1 specific siRNA and western blot was performed on whole cell lysates using an anti-trb-1 antibody.

Figure 2. trb-1 function in hASMC proliferation, migration and chemotaxis. hASMC cells were transfected with a trb-1 overexpression construct **(A)** or si-trb1-1 siRNA **(B)**. Proliferation rate was measured by ^{3}H thymidine incorporation. As an independent measure of proliferation, time-lapse video microscopy was performed and the percentage of mitotic cells on each field were calculated **(C)**. The number of cells migrated through the edge of the wound **(D)** and their speed of migration **(E)** was assessed in a wound-healing assay. **(F)** The effect of MAPK inhibitors on proliferation rate was measured as on panel B. (**G)** The number of cells migrated through the *Boyden chamber* in response to PDGF were compared between control and si-trb-1 transfected cells. In addition, the impact of JNK inhibitor on the migrating cells was also investigated. The impact of tribbles-1 knockdown on the expression of TNFα mRNA **(H)** and TGFβ protein levels **(I)** was evaluated by qRT-PCR and ELISA, respectively.

Figure 3. Overexpression and suppression of trb-1 expression modulates activation of MAPK. (**A)** hASMC cells were transfected with AP-1 luciferase reporter, activated by the co-expression of pFC MEKK1 (both Stratagene) in the presence and absence of overexpressed trb-1, as indicated. (**B)** cells were transfected with empty vector (mock), trb-1 overexpression construct or si-trb1-1 siRNA, stimulated with LPS for 0-45 minutes, as indicated on the figure, lysed and pMAPK and β-actin levels were determined by Western blotting. **(C)** Unstimulaed cell lysates (0 time point) were used to detect the impact of altered trb-1 levels on steady state MAPK expression by Western blotting for total MAPK and β-actin. The signal intensity was quantified as above and expressed as a total MAPK/β-actin ratio. MAPK/β-actin levels in the mock transfected cells were taken as baseline (1 unit) and values measured in the si-trb-1 and overexpressed samples were plotted relative to these.

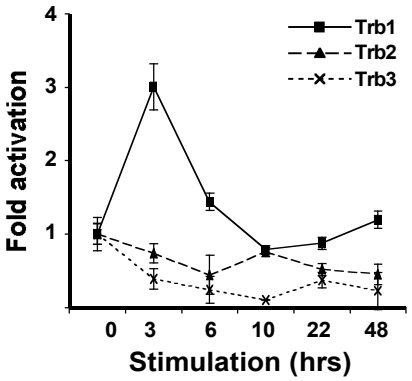
Figure 4. MKK4 - trb-1 interaction controls hASMC proliferation. (A) Expression of MKK4 and MKK7 in hASMC and the impact of trb-1 knockdown on the protein levels of these MAPKKs were assessed by Western Blotting. **(B)** Activation of MKK4 (30 mins, 100ng/ml LPS) was evaluated under normal and reduced trb-1 levels. pMKK4 values were normalised to actin and expressed as a ratio. **(C)** The ability of MKK4 to control hASMC proliferation rate was measured as on Figure 2 (24hrs post-transfection). The efficiency of MKK4 knockdown was verified by western blot (Upper panel) **(D)** Physical interaction between MKK4 and trb-1 in hASMC was investigated by PCA. As positive controls, EGFP expression plasmid (left upper panel) and 'zipper-PCA' (left lower panel, zip-V1 and zip-V2) constructs were used. MKK4 was fused to the N-terminal fragment of Venus-YFP (V1), whilst trb-1 was expressed in fusion with the C-terminal fragment of Venus-YFP (V2). Representative cells show interaction between MKK4 and trb-1 (right panels). **(E)** To further confirm association between trb-1 and MKK4, co-immunoprecipitation was performed, using trb-1-myc expression construct. Lane 1: detection of MKK4 in a whole cell lysate. Lane 2: detection of MKK4 after anti-myc pull-down. **(F)** The impact of the N- and C-terminal domains of trb-1 on interaction with MKK4 in live cells and the location of the trb-1/MKK4 complex was assessed by PCA. **(G)** The structure of trb-1 mutants and the positions of the N- and C-terminal deletions is shown. **(H)** To confirm the specificity of MKK4/trb-1 interaction, FACS was used to demonstrate the specific interaction between trb-1 and MKK4 in HeLa cells. Similarly to figure 5E, an increasing dose of non-fluorescent trb-1 expression plasmid was co-transfected to compete out the labelled protein from the fluorescent complex. Further, no interaction was detected between control plasmids and either MKK4-V1 or trb-1-V2. **(I)** As a further control, an increasing dose of trb-1 expression plasmid (unlabelled) was co-transfected in HeLa cells with the above two constructs (left) and the average total fluorescence per cell was measured (right) by fluorescent microscopy.

Figure 5. trb-1 expression is up-regulated in IHD (A) Total RNA was prepared from sections of human coronary arteries from explanted hearts with IHD (n=8) ("Disease") or DCM (n=6) ("Control"). qRT-PCR was performed to quantify expression levels of major inflammatory cytokines and tribbles 1-3. Expression data was normalised for GAPDH as housekeeping control. Statistical analysis was performed by PRISM, using Student's t-test. Relative expression values are presented using "box and whisker plots". **(B)** Transverse sections of human coronary artery stained with trb-1 antibody. a) Haematoxylin and Eosin staining to show vessel architecture I intima, IEL, internal elastic lamina, M media; b) Trb-1 (brown staining) in intimal and medial areas, boxed area shown in d); c) control staining of a serial section with no primary trb-1 antibody; d) trb-1 staining to show individually stained VSMC (arrows).In a and b scale bar is 50μm; c and d scale bar is 25μm.

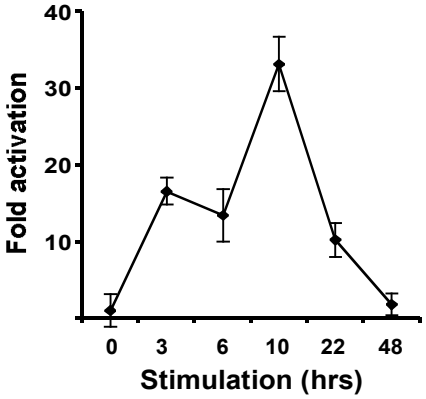
Figure 6. The role of trb-1 in VSMC biology

Figure 1

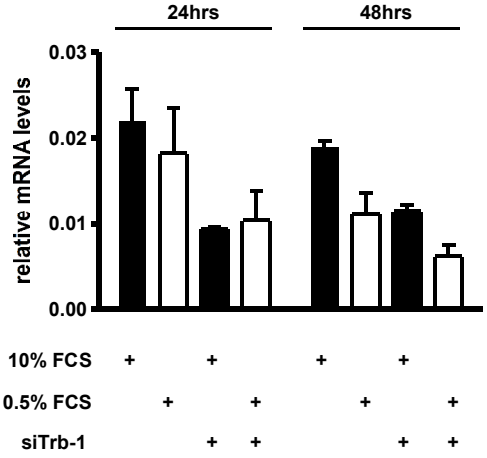
A



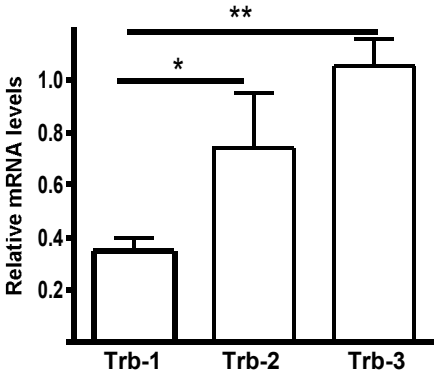
B



C



D



E

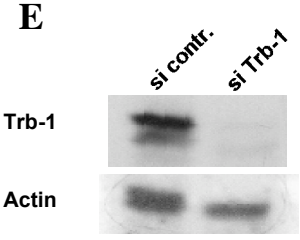


Figure 2

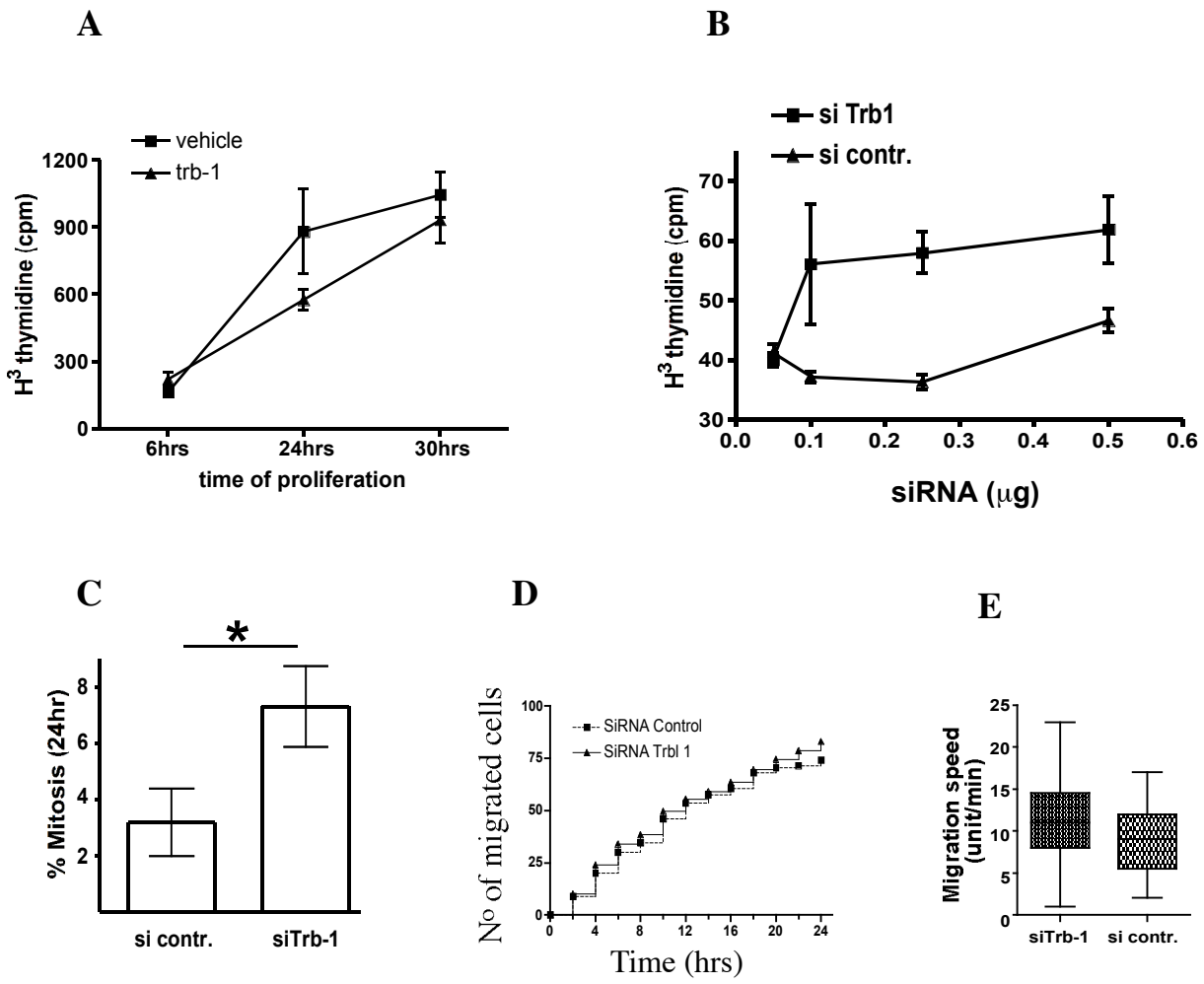
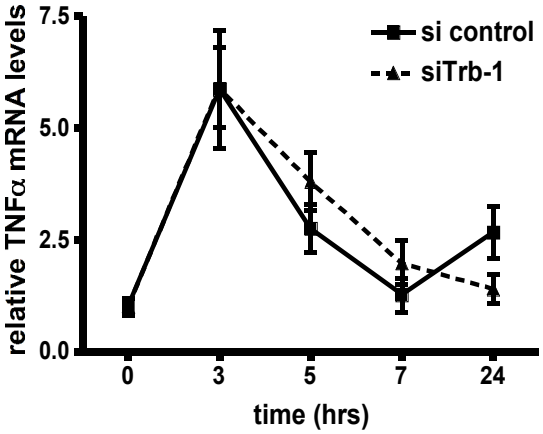

A
vehicle
trb-1
H3 thymidine (cpm)
1200
900
600
300
0
6hrs
24hrs
30hrs
time of proliferation
B
si Trb1
si contr.
H3 thymidine (cpm)
70
60
50
40
30
0.0
0.1
0.2
0.3
0.4
0.5
0.6
siRNA (μg)
C
% Mitosis (24hr)
8
6
4
2
si contr.
siTrb-1
D
No of migrated cells
SiRNA Control
SiRNA Trbl 1
100
75
50
25
0
0
4
8
12
16
20
24
Time (hrs)
E
Migration speed (unit/min)
25
20
15
10
5
0
siTrb-1
si contr.
F
H3 thymidine (cpm)
10000
7500
5000
2500
0
siTrb-1 + + + +
ERK inh. - + - -
JNK inh. - - + -
p38 inh. - - - +
G
PDGF
PDGF + JNK Inh.
No of transmigrated cells
50
40
30
20
10
0
si contr.
siTrb-1

Figure 2

H


relative TNFα mRNA levels
7.5
5.0
2.5
0.0
si control
siTrb-1
0
3
5
7
24
time (hrs)

I

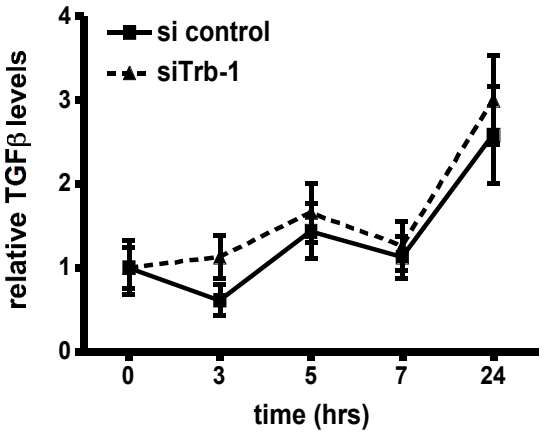

relative TGFβ levels
4
3
2
1
0
si control
siTrb-1
0
3
5
7
24
time (hrs)

Figure 3

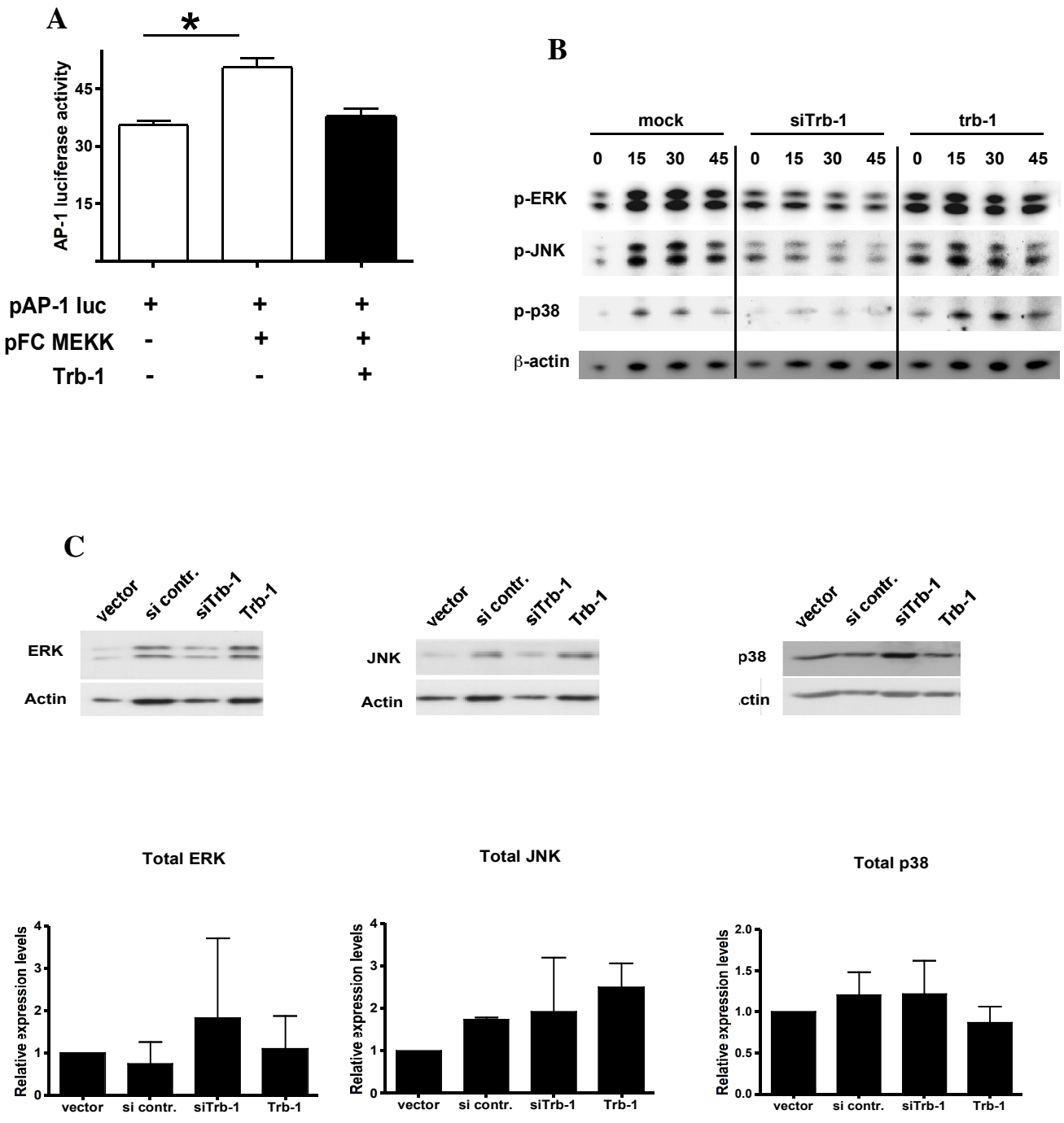
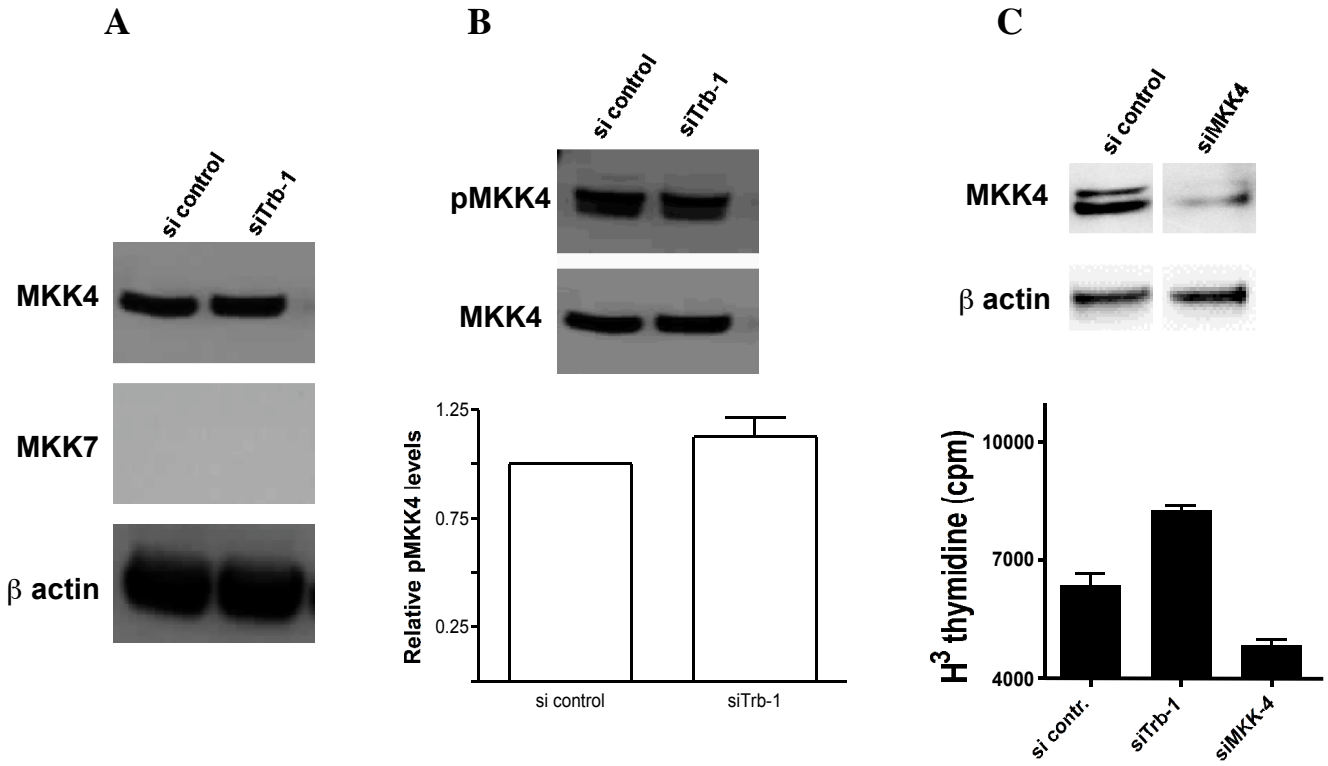
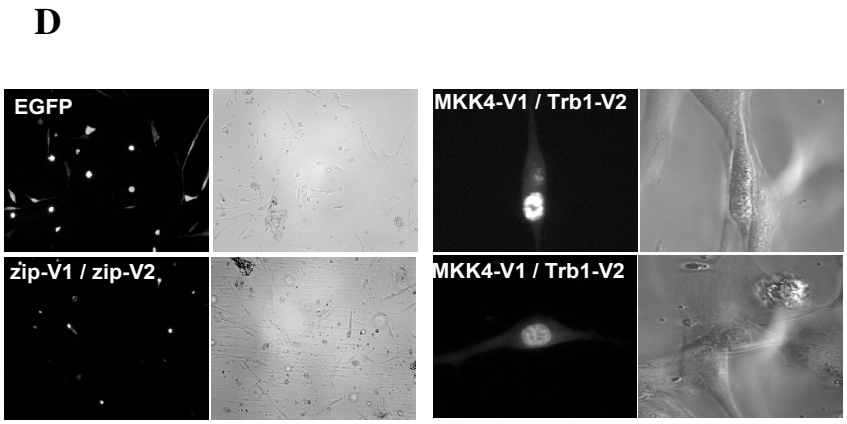
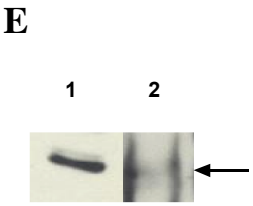

A
*
AP-1 luciferase activity
45
30
15
pAP-1 luc + + +
pFC MEKK - + +
Trb-1 - - +
B
mock
siTrb-1
trb-1
0 15 30 45 0 15 30 45 0 15 30 45
p-ERK
p-JNK
p-p38
β-actin
C
vector
si contr.
siTrb-1
Trb-1
ERK
Actin
JNK
Actin
p38
ctin
Total ERK
Total JNK
Total p38
Relative expression levels
vector
si contr.
siTrb-1
Trb-1

Figure 4


A
si control
siTrb-1
MKK4
MKK7
β actin
B
si control
siTrb-1
pMKK4
MKK4
Relative pMKK4 levels
1.25
0.75
0.25
si control
siTrb-1
C
si control
siMKK4
MKK4
β actin
H3 thymidine (cpm)
10000
7000
4000
si contr.
siTrb-1
siMKK-4


D
EGFP
zip-V1 / zip-V2
MKK4-V1 / Trb1-V2
MKK4-V1 / Trb1-V2


E
1
2

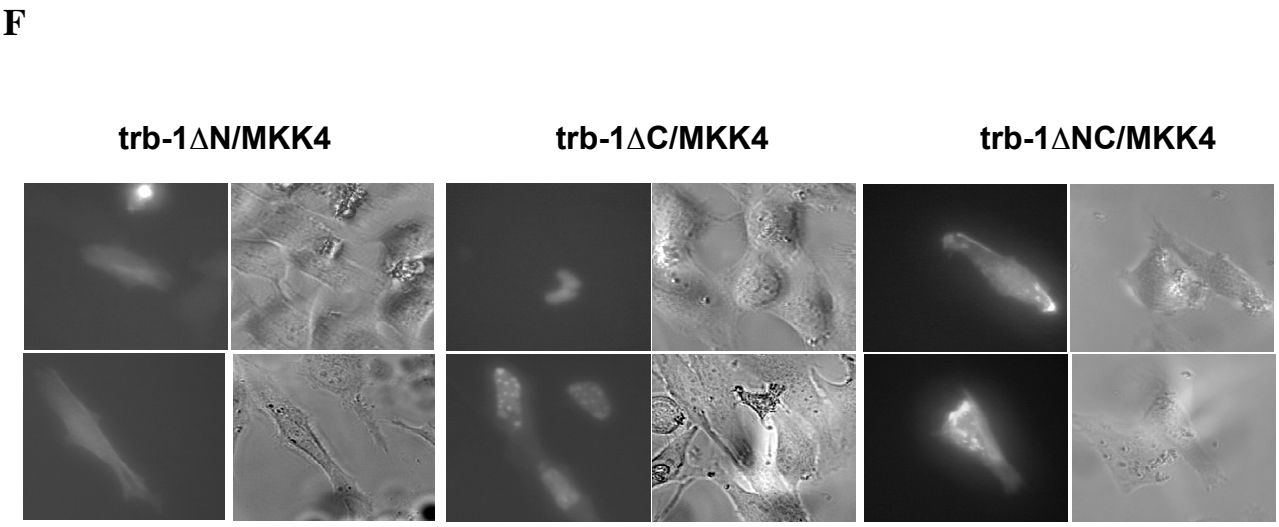
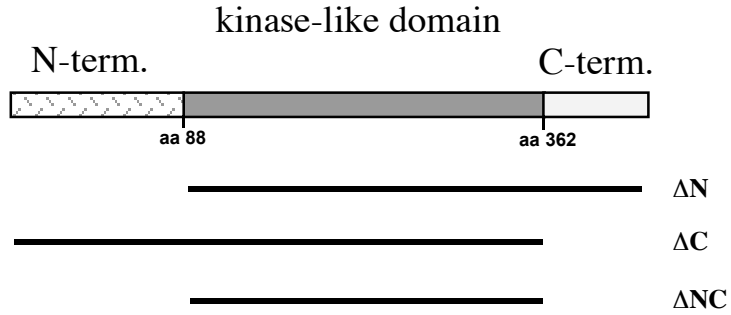
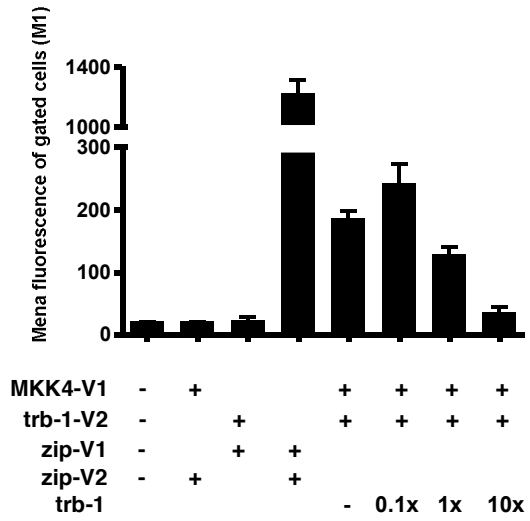

F
trb-1ΔN/MKK4
trb-1ΔC/MKK4
trb-1ΔNC/MKK4

Figure 4

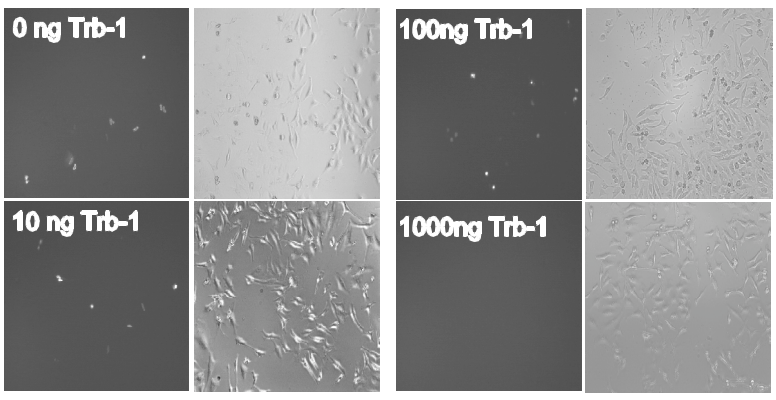
G


kinase-like domain
N-term.
C-term.
aa 88
aa 362
ΔN
ΔC
ΔNC

H


Mena fluorescence of gated cells (M1)
1400
1000
300
200
100
0
MKK4-V1 - + + + + +
trb-1-V2 - + + + + +
zip-V1 - + +
zip-V2 - + +
trb-1 - 0.1x 1x 10x

I


0 ng Trb-1
100ng Trb-1
10 ng Trb-1
1000ng Trb-1

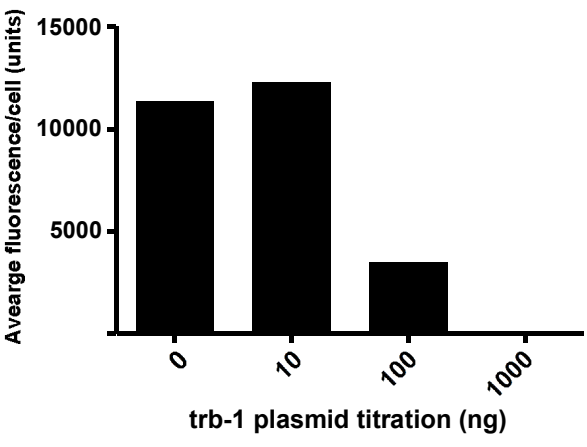
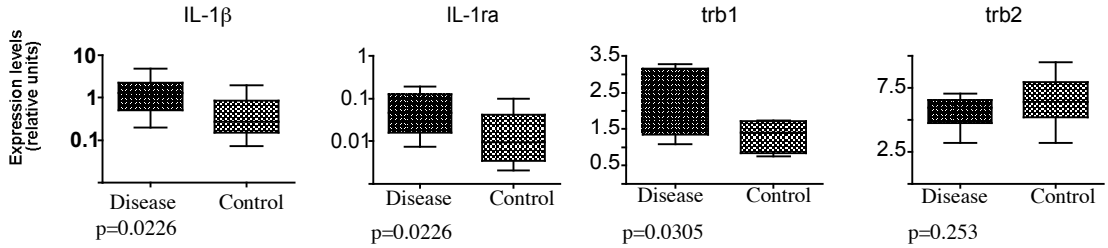

Avearge fluorescence/cell (units)
15000
10000
5000
0
10
100
1000
trb-1 plasmid titration (ng)

Figure 5

A


IL-1β
IL-1ra
trb1
trb2
Expression levels (relative units)
10
1
0.1
1
0.1
0.01
3.5
2.5
1.5
0.5
7.5
2.5
Disease
Control
p=0.0226
p=0.0226
p=0.0305
p=0.253

B

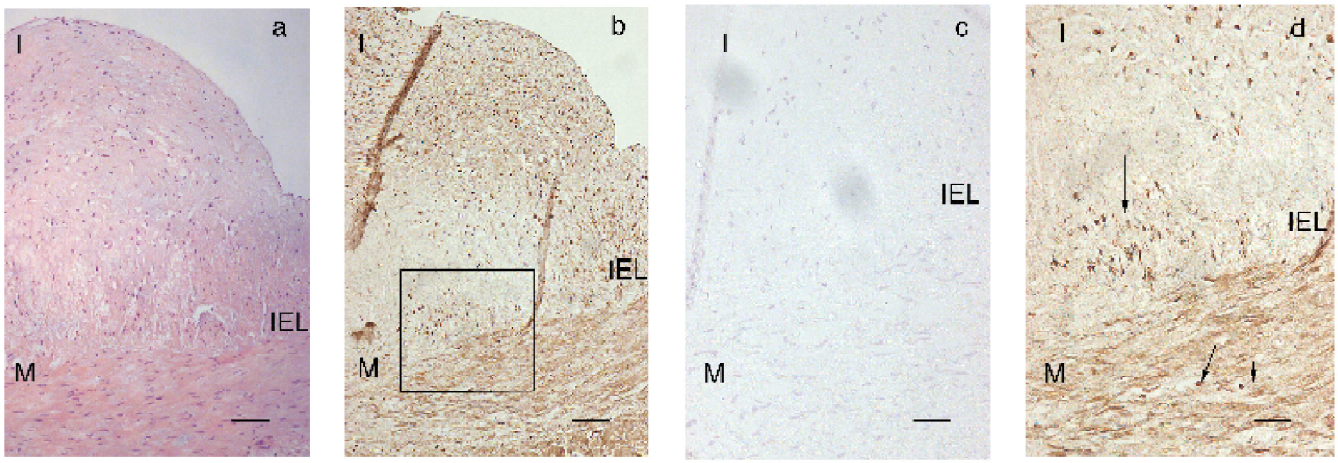
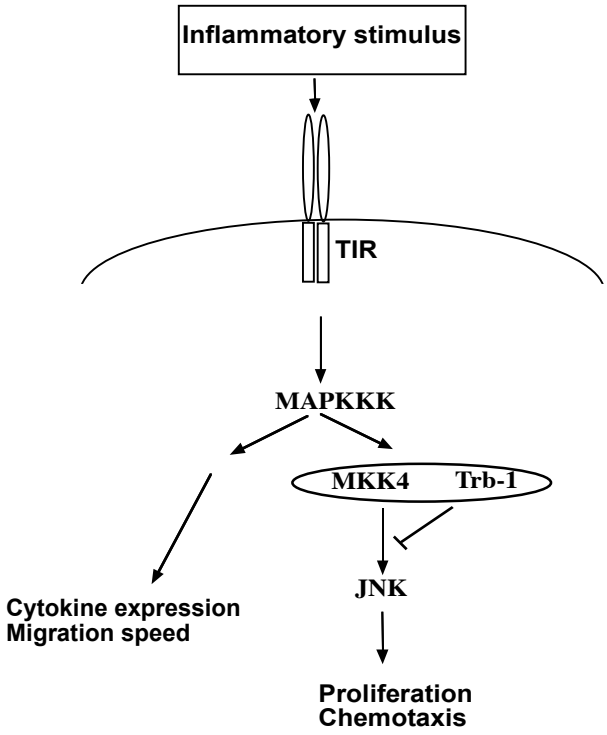

a
b
c
d
I
IEL
M

Figure 6


Inflammatory stimulus
TIR
MAPKKK
MKK4
Trb-1
JNK
Cytokine expression
Migration speed
Proliferation
Chemotaxis