Neurotensin-induced miR-133α expression regulates neurotensin receptor 1 recycling through its downstream target aftiphilin

Ivy Ka Man Law1, Dane Jensen2,3, Nigel W. Bunnett2,3,4 & Charalabos Pothoulakis1

Neurotensin (NT) triggers signaling in human colonic epithelial cells by activating the G protein-coupled receptor, the neurotensin receptor 1 (NTR1). Activated NTR1 traffics from the plasma membrane to early endosomes, and then recycles. Although sustained NT/NTR1 signaling requires efficient NTR1 recycling, little is known about the regulation of NTR1 recycling. We recently showed that NT/NTR1 signaling increases expression of miR-133α. Herein, we studied the mechanism of NT-regulated miR-133α expression and examined the role of miR-133α in intracellular NTR1 trafficking in human NCM460 colonocytes. We found that NT-induced miR-133α upregulation involves the negative transcription regulator, zinc finger E-box binding homeobox 1. Silencing of miR-133α or overexpression of aftiphilin (AFTPH), a binding target of miR-133α, attenuated NTR1 trafficking to plasma membrane in human colonocytes, without affecting NTR1 internalization. We localized AFTPH to early endosomes and the trans-Golgi network (TGN) in unstimulated human colonic epithelial cells. AFTPH overexpression reduced NTR1 localization in early endosomes and increased expression of proteins related to endosomes and the TGN trafficking pathway. AFTPH overexpression and de-acidification of intracellular vesicles increased NTR1 expression. Our results suggest a novel mechanism of GPCR trafficking in human colonic epithelial cells by which a microRNA, miR-133α regulates NTR1 trafficking through its downstream target AFTPH.

Neurotensin receptor 1 (NTR1) is a high affinity G protein-coupled receptor (GPCR) for neurotensin (NT), a 13-amino acid neuropeptide expressed in the central nervous system and the intestine, including ileum and colon. NTR1 is present in colonic epithelial cells and NT/NTR1 coupling in human colonic epithelial cells activates Akt, MAPK, NF-κB pathways. In human colonic epithelial cells the majority of NTR1 is internalized to Rab5a+ early endosomes upon NT exposure and transported back to the plasma membrane to achieve cell re-sensitization. Moreover, we have shown that inhibition of NTR1 recycling from Rab5a+ early endosomes attenuates NT-induced proinflammatory responses in human colonic epithelial cells, suggesting that NTR1 recycling and re-sensitization are required for sustained NT/NTR1 signaling activation in vitro. In different cell types, however, NTR1 is transported to lysosomes for degradation. Therefore, the mechanisms of NTR1 re-sensitization are not well-studied.

MicroRNAs (MiRs) are small, single-stranded RNA molecules which promote translational repression or deadenylation and mRNA degradation. Recently, we have shown that upon NT exposure, miR-133α and other miRs are upregulated in human colonic epithelial NCM460 cells overexpressing NTR1 (NCM460-NTR1). Reducing miR-133α levels in NCM460-NTR1 cells attenuated NT-induced MAPK and NF-κB activation and cytokine production. MiR-133α has been shown to regulate ERK, PI3K/Akt and p53 signaling pathways by directly targeting transcripts related to these pathways. Interestingly, gene silencing of aftiphilin, a novel target of miR-133α associated with intracellular trafficking and secretion, also promotes proinflammatory responses.

1Inflammatory Bowel Disease Center, Division of Digestive Diseases, David Geffen School of Medicine, University of California at Los Angeles, California, USA. 2Monash Institute of Pharmaceutical Sciences, ARC Centre of Excellence in Convergent Bio-Nano Science and Technology Parkville, Monash University, Australia. 3Department of Anesthesia and Peri-operative Medicine, Monash University, Australia. 4Department of Pharmacology and Therapeutics, University of Melbourne, Australia. Correspondence and requests for materials should be addressed to C.P. (email: cpothoulakis@mednet.ucla.edu)
in human colonic epithelial cells. Taken together, we hypothesize that miR-133α mediates NTR1-associated signaling pathways by modulating intracellular NTR1 trafficking. Here we present evidence that the miR-133α/AFTPH axis controls intracellular NTR1 trafficking in human colonic epithelial cells and identified zinc finger E-box binding homeobox 1 (ZEB1) as a negative transcriptional regulator of NT-induced miR-133α expression.

Results

ZEB1 is a negative transcriptional regulator of miR-133α. We have recently reported that incubation of NCM460-NTR1 cells with NT in human colonocytes increases expression of miR-133α. To examine the molecular mechanism by which NT induces miR-133α upregulation, the genomic sequence of 2000 bp upstream to the transcription start site (TSS) of miR-133α was analyzed by the online transcription binding site prediction software, Transcription Element Search System (TESS) (http://www.cbil.upenn.edu/cgi-bin/tess/tess). A transcription binding site (CTGTTTCAC) for ZEB1 was found upstream to miR-133α at position −1150 bp to −1142 bp. We first validated binding of ZEB1 in NT-stimulated human colonic epithelial NCM460-NTR1 cells by immunoprecipitating nuclear extracts from control and NT-exposed cells with a ZEB1 antibody and performing chromatin immunoprecipitation (ChIP). Our results showed ZEB1 binding was reduced upon NT stimulation (Fig. 1A). We next knocked down ZEB1 expression by siRNA in NCM460-NTR1 cells and exposed them to...
miR-133α acts as a negative transcriptional regulator in NT-associated miR-133α expression in response to NT. We constructed. As shown in Fig. 1G, increased miR-133α promoter with deleted ZEB1 binding site. Therefore, ZEB1 was abolished in cells transfected with a miR-133α NC-460-NTR1-AFTPH cells treated with vehicle control (Fig. 2C). Thus, similar to miR-133α reduced presence of NTR1 on the plasma membrane after recovery (1.1±0.18 vs 0.8±0.10, p<0.05), when compared to control as-miR-treated cells (Fig. 2B). These results suggest that knock-down of miR-133α attenuates NTR1 trafficking to plasma membrane in human colonic epithelial cells.

MiR-133α is involved in intracellular trafficking of NTR1 after NT exposure. MiR-133α has been associated with myocyte development and hypertrophy29–30, fibrosis31,32, and oncogenesis20,21,33–37. We recently showed that miR-133α gene silencing attenuates colonic inflammation during experimental colitis, possibly by reducing NT-mediated proinflammatory response in colonic epithelial cells19. NT, through NTR1, activates proinflammatory signaling pathways in colonic epithelial cells7,9,10,13 and this signaling activation is, at least, partially regulated by efficient recycling of NTR1 to the plasma membrane14. Therefore, we hypothesized that NT-upregulated miR-133α might also contribute to intracellular trafficking of NTR1. To localize NTR1 during receptor trafficking in the presence or absence of miR-133α, human colonic epithelial NCM460-NTR1 cells were transfected with antisense (as)-miR-133α or its control and exposed to 100 nM NT (1 hr) in serum-free media to allow NTR1 internalization. The cells were then washed and replenished with NT-free media for 3 hr to allow NTR1 trafficking to the cell surface (recovery). NTR1 cells treated with vehicle control remained on the plasma membrane, while NT (100 nM) exposure internalized NTR1 (Fig. 2A). Importantly, internalization of NTR1 was not affected by miR-133α gene silencing. However, miR-133α downregulation attenuated trafficking of NTR1 to the plasma membrane by retaining NTR1 within intracellular vesicles, while the majority of NTR1 in cells transfected with control as-miR was localized to the plasma membrane 3 hr after removal of NT from the media (Fig. 2A). To confirm this, we performed a biotinylation assay on NCM460-NTR1 cells after recovery, followed by NTR1-specific ELISA to quantify NTR1 localized to the cell surface. As-miR-133α treatment significantly reduced the presence of NTR1 on plasma membrane after recovery (1.0±0.18 vs 0.8±0.10, p<0.05), when compared to control as-miR-treated cells (Fig. 2B). These results suggest that knock-down of miR-133α attenuates NTR1 trafficking to plasma membrane in human colonic epithelial cells.

MiR-133α regulates intracellular trafficking of NTR1 through its binding target, aftiphilin. We have previously identified a novel miR-133α binding target, aftiphilin (AFTPH), which is downregulated following NT exposure of human colonic epithelial NCM460-NTR1 cells19. The function of AFTPH in intestinal epithelial cells is not known. Previous studies suggested that AFTPH knock-down promoted the recycling of endocytosed transferrin to cell periphery in HeLa cells26 and dysregulated exocytosis of Weibel-Palade bodies in endothelial cells without affecting the process of maturation of the granules27. To examine the function of AFTPH in intracellular trafficking of NTR1, NCM460-NTR1 cells overexpressing lentivirus–transduced AFTPH (NCM460-NTR1-AFTPH) were generated and exposed to NT (100 nM) followed by recovery for 3 hr. RT-PCR analysis showed that AFTPH expression in NCM460-NTR1-AFTPH cells was significantly increased (Suppl. Fig. 1, p<0.05). Furthermore, since exogenous AFTPH lacks the original 3’ UTR, its expression level should not be reduced upon NT stimulation. We found that NTR1 was localized on the plasma membrane of NCM460-NTR1-AFTPH cells treated with vehicle control (Fig. 2C). Thus, similar to miR-133α silencing, overexpression of AFTPH did not affect NTR1 internalization or expression of NTR1 on the plasma membrane. However, without AFTPH downregulation during NT exposure, trafficking of NTR1 to the plasma membrane was attenuated when compared to NCM460-NTR1 cells (Fig. 2C). Results from biotinylation assays also confirmed that reduced presence of NTR1 on the plasma membrane after recovery (1.1±0.10 vs 0.6±0.22, p<0.05), when compared to NCM460-NTR1 cells (Fig. 2D). In addition, NTR1 internalization and recycling were also examined in NCM460-NTR1 cells transfected with si-AFTPH and its scrambled si-control. As expected, NTR1 (localized in intracellular vesicles) was further reduced 3 hr after recovery compared to si-control transfected cells (Suppl. Fig. 2A). Our biotinylation assay also showed that membrane-bound NTR1 was increased in si-AFTPH-transfected cells after recovery (1.3±0.61 vs 2.8±0.58, p<0.05) when compared to si-control-transfected cells (Suppl. Fig. 2B). Taken together, absence of AFTPH downregulation during NT exposure attenuates NTR1 trafficking to the plasma membrane in human colonic epithelial cells.

AFTPH overexpression modulates intracellular NTR1 trafficking upon NT exposure. Since as discussed above AFTPH is a novel miR-133α downstream target in human colonic epithelial cells exposed to NT19, we examined the role of AFTPH in NTR1 trafficking in NCM460-NTR1 cells. AFTPH has been localized in TGN25 and early endosomes26 in different cell types. In the present study, without NT stimulation, immunocytochemical analysis showed that a small number of AFTPH exhibited cytosolic localization and co-localized with EEA1, a marker for early endosomes (Fig. 3A). However, the majority of AFTPH displayed a peri-nuclear
localization and was co-localized with the TGN markers golgin 97 and TGN38, (Fig. 3B,C). These results suggest that AFTPH is localized in both early endosomes and TGN in non-stimulated human colonic epithelial cells.

We have shown that translocation of NTR1 to early endosomes is essential for internalized NTR1 to be trans-ported back to the plasma membrane 14. In addition, AFTPH knock-down prevents trafficking of internalized transferrin from early endosomes to recycling endosomes, and thus leads to the accumulation of transferrin in early endosomes 26. Therefore, we next examined whether AFTPH overexpression affected NTR1 translo-cation to early endosomes. NCM460-NTR1 and NCM460-NTR1-AFTPH cells were exposed to NT (100 nM, 1 hr) and NTR1 and EEA1 were localized by immunocytochemistry. NTR1 was localized on the plasma mem-brane under unstimulated condition and internalized upon stimulation in both cell types (Fig. 4A). NTR1 was localized on the plasma membrane under unstimulated condition and internalized upon stimulation in both cell types (Fig. 4A). NTR1/EEA1 co-localization under both conditions in the two cell lines was then quantified by analyzing the images from

---

**Figure 2.** MiR-133α/AFTPH axis regulated NTR1 trafficking to plasma membrane during recovery in human colonic epithelial cells. (A) NCM460-NTR1 cells transfected with as-miR-133α and as-miR-control were exposed to NT (100 nM, 1 hr), washed and replenished with NT-free media (recovery). Trafficking of NTR1 to plasma membrane was attenuated in miR-133α knocked-down NCM460-NTR1 cells, as visualized using immunocytochemistry, (arrow: membrane-bound; arrowhead: vesicle-bound) Scale bars, 10 μm (B) Reduced membrane-bound NTR1 was present in the miR-133α-silenced cells after recovery when compared with control cells, as measured in biotinylation assay. *p < 0.05 when compared to as-miR-control-transfected cells. (C) NCM460-NTR1 (NTR1) cells and cells overexpressing NTR1 and AFTPH (NTR1-AFTPH) were exposed to NT, washed and allowed to recover as described in (A). Trafficking of NTR1 to plasma membrane was attenuated in NCM460-NTR1-AFTPH cells as visualized using immunocytochemistry when compared with NCM460-NTR1 cells. (arrow: membrane-bound; arrowhead: vesicle-bound) Scale bars, 10 μm (D) Reduced membrane-bound NTR1 was observed after recovery in NCM460-NTR1-AFTPH cells when compared to NCM460-NTR1 cells in biotinylation assay. *p < 0.05 when compared to NCM460-NTR1 cells.
the immunocytochemistry experiments. As expected, NT exposure increased NTR1/EEA1 co-localization in both cell lines when compared to unstimulated cells (Fig. 4B, \( p < 0.001 \)). More importantly, AFTPH overexpression reduced co-localization of NTR1 with EEA1 (0.20 ± 0.04 vs 0.17 ± 0.04, \( p < 0.05 \)) when compared to NCM460-NTR1 cells (Fig. 4B). Therefore, overexpression of AFTPH, a TGN-localized protein, reduces NTR1 translocation to early endosomes during NT stimulation.

**MiR-133\( \alpha \)/AFTPH axis modulates expression of proteins in endosomes and TGN.** Next, we studied the effect of miR-133\( \alpha \) and AFTPH overexpression on expression of proteins along endosome trafficking pathways. Transiently miR-133\( \alpha \) gene-silenced NCM460-NTR1 cells and NCM460-NTR1-AFTPH cells were fixed and expression of rab5 (early endosome), rab7 (late endosome), and rab11 (recycling endosome) were determined by In-cell ELISA and compared with their respective controls. We first validated this protein quantification method by showing miR-133\( \alpha \) gene-silencing and AFTPH overexpression increased AFTPH expression (Fig. 5A). Next, we showed that rab7 and rab5 expression were increased upon miR-133\( \alpha \) gene-silencing and AFTPH overexpression, respectively (Fig. 5B,C). More importantly, rab11 expression was significantly increased in both NCM460-NTR1 cells transfected with as-miR-133\( \alpha \) and NCM460-NTR1-AFTPH cells when compared to their respective controls (Fig. 5D). These data suggest that the miR-133\( \alpha \)/AFTPH axis modulates the expression of rab11 from recycling endosomes. However, expression of other endosome markers was also increased, but...
the results were not statistically significant (Fig. 5B,C). The differential expression levels of rab5 and rab7 might be due to differential regulation of expression of genes targeted by miR-133α, other than AFTPH.

Since AFTPH was also localized in TGN (Fig. 3), we next studied the expression of TGN markers in human colonic epithelial cells. Results from In-cell ELISA suggested that expression of golgin97 and TGN38 was significantly increased upon miR-133α gene-silencing and AFTPH overexpression, respectively (Fig. 5E,F). Thus, miR-133α knock-down and AFTPH overexpression results in increased expression of markers of recycling endosomes and TGN.

Figure 4. AFTPH overexpression reduced NTR1 localization in early endosomes upon NT stimulation in human colonic epithelial cells. (A) Cells with AFTPH overexpression (NTR1-AFTPH) showed reduced co-localization of internalized NTR1 and EEA-1, an early endosome marker, upon NT stimulation (100 nM, 1 hr), when compared with NCM460-NTR1 cells as examined in immunocytochemistry. Scale bars, 10 μm. (B) Co-localization of NTR1 and EEA1 was increased upon NT stimulation in both cell lines when compared with the unstimulated condition, as examined by quantitative image analysis. However, co-localization of internalized NTR1 and EEA1 was reduced in NCM460-NTR1 cells overexpressing AFTPH upon NT stimulation when compared with NCM460-NTR1 cells. ***p < 0.005, ****p < 0.0001 when compared to cells in unstimulated conditions. #p < 0.05 when compared to NCM460-NTR1 cells without AFTPH overexpression.
MiR-133α/AFTPH axis acts on a Bafilomycin A1-sensitive trafficking network. We have shown that miR-133α knock-down and AFTPH overexpression retained NTR1 in the cytosol (Fig. 1) during recovery and upregulated protein expression in endosomes and TGN (Fig. 5). On the other hand, studies in other cell types have suggested that NTR1 undergoes lysosome degradation. Therefore, we next examined whether miR-133α or AFTPH overexpression contributed to NTR1 degradation by examining NTR1 levels in NCM460-NTR1 cells with either silenced miR-133α or overexpressing AFTPH. Cells were exposed to NT (100 nM, 1 hr), allowed to recover in NT-free media for 3 hr, and then fixed for NTR1 levels quantification by In-cell ELISA. Our results suggested that miR-133α and AFTPH levels did not affect NTR1 levels in either unstimulated cells or in cells

Figure 5. MiR-133α/AFTPH axis regulated expression of proteins related to endosome and trans-Golgi network trafficking pathways. (A) NCM460-NTR1 cells transfected with as-miR-133α and NCM460-NTR1-AFTPH cells showed increased AFTPH expression in In-cell ELISA assay, when compared to their respective controls. (B) Rab5 expression was increased in NCM460-NTR1-AFTPH cells, when compared to NCM460-NTR1 cells. (C) Rab7 expression was increased in miR-133α gene-silenced NCM460-NTR1 cells when compared to the controls. (D) Rab11 expression was increased in both NCM460 cells transfected with as-miR-133α and NCM460-NTR1-AFTPH cells when compared to their respective controls. (E) Increase in TGN38 expression was observed in NCM460-NTR1-AFTPH cells when compared with controls. (F) Golgin97 expression was increased in NCM460-NTR1 cells transfected with as-miR-133α, when compared with controls. *p < 0.05, **p < 0.01, ***p < 0.005 when compared with respective controls.
after recovery from NT exposure (Fig. 6A). Our findings suggest that AFTPH overexpression during recovery promotes NTR1 retention in the cytosol, without affecting NTR1 degradation in human colonic epithelial cells.

Next, we examined whether attenuation of NTR1 trafficking to cell membrane was related to de-acidification of intracellular vesicles or impaired TGN functions. Bafilomycin A1 (BafA1, 100 nM), a specific vacuolar H⁺-ATPase inhibitor, increased NTR1 levels in as-miR-133α-transfected or AFTPH-overexpressing cells when compared to their respective controls. Similar effects were not observed in cells treated with Brefeldin A (BFA, 5 μg/mL). **p < 0.01 when compared with cells without incubating with inhibitors during recovery. (C) Bafilomycin A1 and Brefeldin A treatment during recovery blocked NTR1 recycling in both NCM460-NTR1 cells and their AFTPH-overexpressing counterparts when compared to their respective controls, as visualized in immunocytochemistry. Scale bars, 10 μm. (D) Intracellular NTR1 expression was increased in NCM460-NTR1-AFTPH cells treated with Bafilomycin A1, when compared to NCM460-NTR1 cells under the same treatment as examined in quantitative image analysis. Incubation with Brefeldin A during recovery did not generate similar effect on the two cell lines. *p < 0.05 when compared with cells treated with vehicle controls.

Figure 6. AFTPH overexpression increased NTR1 levels in human colonic epithelial cells treated by Bafilomycin A1. (A) MiR-133α gene silencing and AFTPH overexpression in NCM460-NTR1 cells did not affect NTR1 levels in human colonic epithelial cells when compared with their respective controls under the same treatment (unstimulated or upon 3 hr after recovery), as examined by In-cell ELISA. (B) Treatment with Bafilomycin A1 (BafA1, 100 nM), a specific vacuolar H⁺-ATPase inhibitor, increased NTR1 levels in as-miR-133α-transfected or AFTPH-overexpressing cells when compared to their respective controls. Similar effects were not observed in cells treated with Brefeldin A (BFA, 5 μg/mL). **p < 0.01 when compared with cells without incubating with inhibitors during recovery. (C) Bafilomycin A1 and Brefeldin A treatment during recovery blocked NTR1 recycling in both NCM460-NTR1 cells and their AFTPH-overexpressing counterparts when compared to their respective controls, as visualized in immunocytochemistry. Scale bars, 10 μm. (D) Intracellular NTR1 expression was increased in NCM460-NTR1-AFTPH cells treated with Bafilomycin A1, when compared to NCM460-NTR1 cells under the same treatment as examined in quantitative image analysis. Incubation with Brefeldin A during recovery did not generate similar effect on the two cell lines. *p < 0.05 when compared with cells treated with vehicle controls.
unknown. Here, we have treated as-miR-133α-transfected NCM460-NTR1 cells and NCM460-NTR1-AFTPH cells and their respective controls with BafA1 (100 nM) and Brefeldin A (BFA, 5 μg/mL) during recovery. BFA is a fungal metabolite redistributing TGN proteins to endoplasmic reticulum and early endosomes depending on the cell type46,47. DMSO alone, the solvent for BafA1 and BFA, was used as a control treatment. Expression levels and intracellular distribution of NTR1 in NCM460-NTR1 cells with or without AFTPH overexpression were measured and visualized by In-cell ELISA and immunocytochemistry, respectively. As shown in Fig. 6B, BafA1 and BFA treatment did not alter NTR1 levels in NCM460-NTR1 cells transfected with scrambled control or in non-transfected NCM460-NTR1 cells after recovery. However, BafA1 treatment during recovery increased NTR1 levels in miR-133α gene-silenced NCM460-NTR1 cells and NCM460-NTR1-AFTPH cells (Fig. 6B). BFA treatment did not alter NTR1 levels in any experimental group. We further verified this observation with immunocytochemistry in NCM460-NTR1 cells and NCM460-NTR1-AFTPH cells using NTR1-specific antibodies. Our results showed that the majority of NTR1 in NCM460-NTR1 cells were present at the plasma membrane, while those in AFTPH-overexpressing cells were partially retained in the cytosol (Fig. 6C). On the other hand, NTR1 recycling was largely inhibited by BFA treatment in both cell lines (Fig. 6C). BafA1 treatment also inhibited NTR1 recycling in both cell lines, similar to our previous observation14 (Fig. 6C). In addition, our results suggested that NTR1 aggregated in the cytosol under BafA1 treatment during recovery in NCM460-NTR1-AFTPH cells, but not in NCM460-NTR1 cells (Fig. 6C). Our results suggested failure to reduce endogenous AFTPH levels after NT stimulation caused NTR1 retention in cytoplasm, which coincided with increased Rab11 expression in endosome trafficking pathway.

Discussion

NT activates proliferative8,9,11,12 and pro-inflammatory7,9,10,13 signaling pathways in human colonic epithelial cells through its high affinity GPCR, NTR11. Recently, we have shown that NT/NTR1 coupling increases miR-133α levels in human colonic epithelial cells and knock-down of miR-133α downregulates NT-induced proinflammatory signaling in vitro8 through its downstream target AFTPH19. On the other hand, we have also shown that sustained NTR1 signaling is associated with efficient trafficking of NTR1 to the cell surface after NT stimulation in human colonic epithelial cells14. Taken together, these results suggest that NT-induced miR-133α expression may be involved in NTR1 trafficking in vitro. In this current study, we show that miR-133α expression is negatively regulated by the transcription factor ZEB1 via a ZEB1 binding site on its promoter upon NT stimulation in human colonic epithelial cells. We further showed that the NT-driven miR-133α/AFTPH axis regulates NTR1 trafficking and expression of proteins involved in endosomal trafficking and NTR1 intracellular destination following ligand exposure (Fig. 7).

Using a combination of bioinformatic analysis, RT-PCR, ChiP and luciferase assays, we identified ZEB1 as a NT-driven negative transcriptional regulator of miR-133α in human colonic epithelial cells (Fig. 1). Our results reveal that ZEB1 directly binds to the miR-133α promoter region and suppresses miR-133α expression acting...
translocate between endosomes and TGN56–59, therefore, our results suggest AFTPH overexpression modulates colonocytes (Fig. 5) without affecting NTR1 expression levels during recovery (Fig. 6A). TGN38 and golgin97 increased expression of rab11 (recycling endosome marker), and TGN38 and golgin97 (TGN markers) in human erythroblast HEL cells 48. The role of ZEB1 as transcription repressor in the present study supports its putative role as a transcriptional repressor during epithelial-mesenchymal transition (EMT)50,51 and dedifferentiation 31. Of interest, colon epithelial miR-133α expression is associated with colon cancer development 20,21,24,33–35. Since high NTR1 expression was observed in both inflamed colon tissues 4–6 and in colonic cancer cells 8, suppression of miR-133α expression by ZEB1/miR-133α binding in human colonic epithelial cells may play a role in the NTR1/miR-133α interactions during colonic inflammation and early cancer development through NTR1 re-sensitization in vitro and in vivo.

Although intracellular NTR1 trafficking has been studied extensively 14–16,52, the role of miRs in trafficking of NTR1 or other GPCRs has not been studied. We show that blocking NT-induced miR-133α upregulation in human colon epithelial cells increases NTR1 retention in the cytoplasm as observed by confocal microscopy and biotinylation assays (Fig. 2A,B). Moreover, we have also shown that overexpression of AFTPH, a miR-133α target 19, also attenuates NTR1 trafficking back to plasma membrane after NT exposure (Fig. 2C,D). Our data suggested that since miRs regulate mRNA stability 53 and protein translation 54,55, miR expression induced by GPCR activation may present a novel regulatory pathway for GPCR recycling on an epigenetic level.

Previous studies demonstrate that AFTPH is localized in clathrin-coated vesicles (CCV) 25,26, TGN 25 and early endosomes 26 in neurons and HeLa cells. Our present study extended these observations by showing that the majority of AFTPH are localized in the TGN in human colon epithelial cells (Fig. 3B,C). Furthermore, AFTPH is also localized in the early endosomes (Fig. 3A), which may be explained by the interaction of AFTPH with the AP-1 complex, which is localized in endosomes 25,26. Functionally, AFTPH is associated with exocytosis in endothelial cells 27. During transferrin recycling, knocking down AFTPH promotes the transport of internalized transferrin to early endosomes 26. In our study, the localization of NTR1 remains cytosolic after recovery in human colon epithelial cells overexpressing AFTPH (Fig. 2C,D) while AFTPH knocking down promotes trafficking of NTR1 to the plasma membrane (Suppl. Fig. 2). More importantly, AFTPH overexpression reduced the co-localization of internalized NTR1 and EEA1, an early endosome marker (Fig. 4), in human colon epithelial cells. The above observations and those in transferrin recycling 26 suggest that AFTPH knock-down induced by NTR1/miR-133α regulation 19 suppresses the trafficking of NTR1 from early endosomes to recycling endosomes, thus promoting the rapid recycling by NTR1 from early endosomes back to the plasma membrane 26. Cell desensitization to a ligand-stimulant depends on the efficiency of receptor internalization, while cell re-sensitization is partially achieved by transporting unbound receptors back to plasma membrane. In human colon epithelial cells, NT-induced proinflammatory responses are attenuated by downregulation of miR-133α, but promoted by AFTPH gene silencing 39. Therefore, part of NT/NTR1 signaling might promote trafficking of internalized NTR1 to the plasma membrane through a miR-133α/AFTPH axis, leading to NTR1 re-sensitization.

In addition, AFTPH overexpression by both miR-133α knock-down and stable AFTPH overexpression increased expression of rab11 (recycling endosome marker), and TGN38 and golgin97 (TGN markers) in human colonocytes (Fig. 5) without affecting NTR1 expression levels during recovery (Fig. 6A). TGN38 and golgin97 translocate between endosomes and TGN 56–59, therefore, our results suggest AFTPH overexpression modulates NTR1 trafficking through upregulating expression of proteins involved in trafficking between endosomes and TGN during recovery. We also demonstrated the importance of TGN to NTR1 trafficking to the plasma membrane during recovery by showing NTR1 retention in cytoplasm in human colon epithelial cells with TGN structure and function disrupted by BFA treatment 47,60 (Fig. 6C). Interestingly, BFA-induced inhibition of NTR1 trafficking to the plasma membrane during recovery did not affect NTR1 expression in human colon epithelial cells regardless of AFTPH expression levels. However, de-acidification of endosomes and TGN by BafA1, a specific vacuolar H+-ATPase inhibitor 39, increased NTR1 levels in cells overexpressing AFTPH (Fig. 6B,C). Since the low pH environment of TGN and recycling endosomes is maintained by functional H+-ATPase 44, the accumulation of NTR1 in AFTPH-overexpressing cells suggests that AFTPH may be associated with NTR1 degradation when endosome and TGN transport are impeded. Of note, loss of endosomal acidification may also prevent NTR1 recycling by attenuating the dissociation of NT/NTR1 complex in early endosomes 14, which, in turn, leads to dysregulation in NTR1 recycling. In contrast, since BFA redistributes TGN vesicles to endoplasmic reticulum and early endosomes 56,47, our results imply that AFTPH may not be involved in structural maintenance of TGN in human colon epithelial cells, as observed in neurons 25.

In summary, NT/NTR1 coupling induces the dissociation of ZEB1, a negative transcription regulator, to miR-133α promoter, leading to upregulation of miR-133α. MiR-133α and its downstream target AFTPH, in turn, regulate the intracellular trafficking of the internalized NTR1 and thereby regulating the NTR1 recycling efficiency and re-sensitization in human colon epithelial cells. AFTPH overexpression increases expression of proteins participating in endosome and TGN transport (Fig. 7). Our results suggest that NT/ZEB1/miR-133α/AFTPH signaling may represent a novel epigenetic regulatory network involved in NTR1 trafficking.

Methods

Materials. NT was from Bachem Americas, Inc (Torrance, CA). Bafilomycin A1 and Brefeldin A (both dissolved in DMSO) were from Santa Cruz Biotechnology. Cell culture medium M3:D was from INCELL Corp. (San Antonio, TX). Antisense-miR-133α (as-miR-133α) and its negative control (as-miR-control), TaqMan® probe against AFTPH and ZEB1, TaqMan® Universal PCR Master Mix (2×), Lipofectamine 2000, Lipofectamine™ RNAiMAX, TRIZol, Pierce™ Blocking Buffer were from Life Technologies (Carlsbad, CA). si-RNA against ZEB1 (si-ZEB1), and their negative control (si-Control) were from Santa Cruz Biotechnology. E3-link™ Sulfo-NHS-LC Biotin and Pierce Agarose ChIP kit were from Thermo Scientific (Rockford, IL). Gluthathione was from Fisher Scientific (Rockford, IL). QuikChange II XL site-directed mutagenesis Kit was from Agilent Technologies (Santa...
Clara, CA). Dual-luciferase reporter assay system, pGL3-Basic and pRL-TK were from Promega BioSciences (St. Luis, CA). AFTPH 3′ UTR luciferase reporter plasmid was from Switchgear Genomics (Carlsbad, CA). hsa-miR-133a-5p LNA™ PCR primer set, Universal cDNA Synthesis Kit II and ExiLENT SYBR® Green master mix were from Exiqon ( Vedbaek, Denmark). IRDye® 800 CW Donkey anti-Mouse IgG, IRDye® 680RD Donkey anti-Goat IgG, CellTag™ 700 Stain are from LI-COR Biosciences (Lincoln, NE). Antibodies used in this study include: Goat polyclonal antibodies against AFTPH (sc-167055) and NTR1 (sc-7596); rabbit polyclonal antibodies against early endosomal antigen 1 (EEA1, sc-33585), trans-Golgi network protein 2 (TGN38, sc-33783), Rab5a (sc-309), Rab7 (sc-10767), Rab11 (sc-9020); and mouse monoclonal antibody against Golgi-associated protein golgin A1 (golgin 97, sc-59820) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bovine anti-goat IgG FITC (sc-2348), bovine anti-rabbit IgG-R (sc-2367) and bovine anti-mouse IgG-R (sc-2368) are from Santa Cruz Biotechnology. Paraformaldehyde solution 4% PBS and UltraCruz™ Mounting Medium are from Santa Cruz Biotechnology.

Generation of NCM460-NTR1 and NCM460-NTR1-AFTPH cells. NCM460 cells overexpressing NTR1 (NCM460-NTR1) were generated and maintained as described. NCM460-NTR1-AFTPH cells were generated by transduction of lentivirus carrying full-length human AFTPH gene. The full-length human AFTPH gene was isolated from the original plasmid backbone pCMV6-Entry (Origene) by PCR. The purified AFTPH fragment was isolated from an agarose gel, digested with SpeI and XhoI and in inserted into the multiple cloning site (MCS) of pShuttle CMV-MCS-hrGFP-2 (Clontech) creating a 3′ HA tag at the C-terminus. A PCR fragment of the full length AFTPH-HA was isolated from an agarose gel, digested with SpeI and ligated into the MCS of lentiviral backbone CMV-MCS-ires-Puro to create the lentiviral vector CMV-AFTPH-HA-ires-Puro. Generation of lentivirus particles using the third generation packaging plasmids was as previously described. Briefly, nonconfluent 293T cells were co-transfected with pMDLg/p RRE (gag/pol), pMD.G (encoding the VSV-G envelope), pRSV–REV and CMV-AFTPH-HA-ires-Puro by the CaPO4-DNA coprecipitation method. Viral titer was determined by assessing viral p24 antigen concentration by ELISA (Coulter Immunotech, Miami, FL) and hereafter expressed as μg of p24 equivalent units per milliliter. After transduction, NCM460-NTR1-AFTPH cells were cultured in M3:D complete media supplemented with 10 μg/mL puromycin for selection. Expression of AFTPH in NCM460-NTR1-AFTPH cells was verified by RT-PCR.

Biotinylation assay. Biotinylation of surface NTR1 was achieved by a modification of a previously described method. NCM460-NTR1 cells were washed with PBS and labeled with 1 mg/mL EZ-link® Sulfo-NHS-LC Biotin for 15 min at 4 °C. Unbound biotin was quenched with Tris-buffered saline (TBS) at 4 °C and cells were washed with HBSS, recovered in medium (37 °C for 30 min) and exposed to 100 nM NT for 1 hr at 37 °C. After washing with HBSS, 50 mM glutathione in HBSS was used to strip biotin from proteins remaining at the cell surface. Endocytosed NTR1 was allowed to recycle to plasma membrane in culture medium for 3 hr at 37 °C. Biotin bound to recycled NTR1 was stripped, and unbound biotin was quenched by TBS. Cells were then lysed with 1% Triton X-100 in HBSS supplemented with protease inhibitor cocktail. NTR1 recycling was quantified using NTR1-specific ELISA. Antibodies against NTR1 were used to coat 96-well plate. The plate was blocked in PBS containing 3% bovine serum and 0.1% Triton X and equal amount of lysates were loaded to each well in triplicates and incubated for 16 hr at 4 °C. This was followed by washing with PBS containing 0.05% Tween-20 and incubation with IRDye® 800CW Streptavidin (1 hr, room temperature). After washing, the fluorescent intensity was read by Odyssey® CLX Infrared Imager (LI-COR Biosciences) and quantified by LI-COR® Image Studio (LI-COR Biosciences). Membrane-associated NTR1 was calculated by the equation 1/(biotinylated NTR1 after treatment/Total biotinylated NTR1).

Messenger RNA and microRNA expression analysis. NCM460-NTR1 cells were washed once with ice-cold PBS after various treatments. Total RNA were extracted by TRIzol and reverse-transcribed into first strand cDNAs using random decamers and reverse transcriptase for miRNA expression analysis using TaqMan® Universal PCR Master Mix (2×). Complementary DNAs for microRNA expression analysis were prepared with Universal cDNA Synthesis Kit II and ExiLENT SYBR® Green master mix according to the manufacturer’s instructions.

Cloning and site-directed mutagenesis. The miR-133α promoter-driven luciferase reporter construct (pGL3-miR-133α) was generated by ligating PCR products encoding the genomic region of 2000 bp upstream to miR-133α was XhoI/HindIII digest and pGL3-Basic. The primers used are i) miR-133α XhoI F: cgcctgagttcaagaaataattgctgaa; ii) miR-133α HindIII R: cccaaagcttagtgctgctagtttggaatcc. The following site-directed mutagenesis was done using QuickChange II XL site-directed mutagenesis Kit according to the manufacturer's instructions. ZEB1 binding site on the miR-133α promoter-driven luciferase reporter construct were detected using Dual-luciferase reporter assay system. The relative miR-133α promoter-driven luciferase activities were calculated by normalizing Firefly luciferase activity with that from Renilla luciferase.

Luciferase assays. Plasmids expressing AFTPH 3′ UTR-driven luciferase, pGL3-miR-133α or pGL3-miR-133α AGETB1 and pRL-TK (control) were transfected to NCM460-NTR1 cells using lipofectamine 2000. Two days after transfection NCM460-NTR1 cells were exposed to NT (1 hr), firefly and Renilla luciferase cell activities were detected using Dual-luciferase reporter assay system. The relative miR-133α promoter-driven luciferase activities were calculated by normalizing Firefly luciferase activity with that from Renilla luciferase.

ChIP assay. NCM460-NTR1 cells were cross-linked and fixed after NT exposure for 1 hr using Pierce Agarose ChIP kit according to the manufacturer's instructions. The ZEB1 binding site was immunoprecipitated by rabbit anti-ZEB1 antibody (Bethyl Laboratories). ZEB1 binding was quantified by real time PCR using a primer
complementary to ZEB1 binding site in miR-133α promoter region (Applied Biosystems, assay ID: AJPACV3, Part no. 4441114).

Localization of NTR1, EEA1, golgin 97 and TGN38. NCM460 cells overexpressing NTR1 (NCM460-NTR1) were transfected with as-miR-133α or si-AFTPH and their corresponding controls. NTR1 recycling studies were performed as previously described14. In brief, two days after transfection, NCM460-NTR1 cells were serum-fasted overnight and then exposed to 100 nM NT for 1 hr at 37 °C. NCM460-NTR1 cells were then washed with PBS twice and replenish with NT-free medium. Cells were fixed in PBS containing 4% (w/v) paraformaldehyde (pH 7.4, 20 min, 4 °C) and blocked in PBS containing 3% bovine serum and 0.1% Triton X. Cells were incubated (16 hr, 4 °C) with anti-NTR1 antibodies14 (2 μg/μL), washed and incubated (2 hr, room temperature) with bovine anti-goat-FITC (2 μg/mL). After washing with PBS, cells were mounted with UltraCruz™ Mounting medium.

Co-localization studies were performed as mentioned except that the fixed cells were incubated with two primary antibodies: anti-NTR1/anti-EEA154, anti-AFTPH/anti-EEA1, anti-AFTPH/anti-golgin9795, anti-AFTPH/anti-TGN3894 where appropriate. Bovine anti-rabbit- and bovine anti-mouse-IgG-R were used as secondary antibodies where appropriate.

The stained cells were imaged with a Zeiss LSM 510 Meta laser scanning confocal microscope using a Zeiss 63X Plan-Apo/1.4 oil immersion objective (numerical aperture 1.4). Five Z-stack images were captured using Zen Software (Zeiss). Average NTR1 intensity in cells under different treatments was quantified by the following equation: [NTR1 signal (total – nucleus)/Area (total – nucleus)].

In-cell ELISA. In assays studying the expression levels of endosomal proteins, proteins related to TGN and NTR1, NCM460-NTR1 and NCM460-NTR1-AFTPH cells were seeded and fixed in 96-well culture plates. In assays studying NTR1 expression after recovery, NCM460-NTR1 and NCM460-NTR1-AFTPH cells were stimulated with NT (100 nM, 1 hr) and internalized NTR1 were allowed to recover in NT-free media in the presence of Bafilomycin A1 (100 nM), Brefeldin A (5 μg/mL) and DMSO (solvent control) for 3 hr before fixing. In-cell Western Blot was performed as the manufacturer’s instructions with modifications. Briefly, the cells were permeabilized with 0.1% Triton X-100 in 1 × PBS and blocked with Odyssey® Blocking Buffer (LI-COR) for 1 hr at room temperature. Antibodies against NTR1, AFTPH, Rab5α, Rab7 and Rab11 were diluted in the same blocking buffer (2 μg/mL) and incubated with the cells at 4 °C overnight. After washing with 0.1% Tween 20 in 1 × PBS, secondary antibodies against goat, rabbit and mouse were used as appropriate (2 μg/mL) and CellTag™ 700 Stain (LI-COR) were incubated with the cells at room temperature for 1 hr. NCM460-NTR1 cells were serum-fasted overnight and then exposed to 100 nM NT for 1 hr at 37 °C. NCM460-NTR1 cells were incubated (16 hr, 4 °C) with anti-NTR1 antibodies63, anti-AFTPH19/anti-EEA1, anti-AFTPH/anti-golgin9763; anti-AFTPH/anti-TGN3863 where appropriate.

Mounting medium.

Statistical analysis. All results were derived from at least three sets of experiments, expressed as means ± SD and analyzed with Student’s t-Tests. In all statistical comparisons, p < 0.05 was used to indicate significant differences.

References
1. Tanaka, K., Masu, M. & Nakaniishi, S. Structure and functional expression of the cloned rat neurotensin receptor. Neuron 4, 847–854 (1990).
2. Carraway, R. & Leeman, S. E. Characterization of radioimmunoassayable neurotensin in the rat. Its differential distribution in the central nervous system, small intestine, and stomach. J Biol Chem 251, 7045–7052 (1976).
3. Polak, J. M. et al. Specific localization of neurotensin to the N cell in human intestine by radioimmunoassay and immunocytochemistry. Natur 270, 183–184 (1977).
4. Castagliuolo, I. et al. Neurotensin is a proinflammatory neuropeptide in colonic inflammation. The Journal of clinical investigation 103, 843–849 (1999).
5. Koon, H. W. et al. Neurotensin induces IL-6 secretion in mouse preadipocytes and adipose tissues during 2,4,6-trinitrobenzene sulfonic acid-induced colitis. Proceedings of the National Academy of Sciences of the United States of America 106, 8766–8771 (2009).
6. Brun, P. et al. Neuropeptide neurotensin stimulates intestinal wound healing following chronic intestinal inflammation. Am J Physiol Gastrointest Liver Physiol 288, G621–629 (2005).
7. Zhao, D. et al. Signal transduction pathways mediating neurotensin-stimulated interleukin-8 expression in human colonocytes. J Biol Chem 276, 44464–44471 (2001).
8. Bakirtzi, K. et al. Neurotensin Signaling Activates MicroRNAs-21 and -155 and Akt, Promotes Tumor Growth in Mice, and Is Increased in Human Colon Tumors. Gastroenterology 141, 1769–1776.e1741 (2011).
9. Zhao, D. et al. Insulin-like growth factor-1 receptor transactivation modulates the inflammatory and proliferative responses of neurotensin in human colonic epithelial cells. J Biol Chem 286, 6092–6099 (2011).
10. Zhao, D. et al. Neurotensin stimulates IL-8 expression in human colonic epithelial cells through Rho GTPase-mediated NF-kappa B pathways. American journal of physiology. Cell physiology 284, C1397–1404 (2003).
11. Zhao, D. et al. Metalloprotease-dependent transforming growth factor-alpha release mediates neurotensin-stimulated MAP kinase activation in human colonic epithelial cells. J Biol Chem 279, 43547–43554 (2004).
12. Zhao, D. et al. Neurotensin stimulates expression of early growth response gene-1 and EGF receptor through MAP kinase activation in human colonic epithelial cells. International journal of cancer. Journal international du cancer 120, 1652–1656 (2007).
13. Zhao, D. et al. Neurotensin stimulates interleukin-8 expression through modulation of I kappa B alpha phosphorylation and p65 transcriptional activation: in vivo and in vitro. Mol Pharmacol 67, 2025–2031 (2005).
14. Law, I. K., Murphy, J. E., Bakirtzi, K., Bunnett, N. W. & Pothoulakis, C. Neurotensin-induced proinflammatory signaling in human colonocytes is regulated by beta-arrestins and endothelin-converting enzyme-1-dependent endocytosis and resensitization of neurotensin receptor J. Biol Chem 287, 15066–15075 (2012).
15. Botto, J. M., Chabry, J., Sarret, P., Vincent, J. P. & Mazella, J. Stable expression of the mouse levocabastine-sensitive neurotensin receptor in HEK 293 cell line: binding properties, photoaffinity labeling, and internalization mechanism. Biochem Biophys Res Commun 243, 585–590 (1998).
16. Vandenbulcke, F., Noisel, D., Vincent, J. P., Mazella, J. & Beaudet, A. Ligand-induced internalization of neurotensin in transfected COS-7 cells: differential intracellular trafficking of ligand and receptor. J Cell Sci 113 (Pt 17), 2963–2975 (2000).
17. Hermans, E. & Maloteaux, J. M. Mechanisms of regulation of neurotensin receptors. *Pharmacol Ther* 79, 89–104 (1998).
18. Bartel, D. P. MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215–233 (2009).
19. Law, I. K. M. et al. Neurotensin—regulated mirt-133 prevents myoblast proliferation and differentiation. *Cell death & disease* 4, e934 (2013).
20. Wang, H. et al. mirt-133a represses tumour growth and metastasis in colorectal cancer by targeting LIM and SH3 protein 1 and inhibiting the MAPK pathway. *European journal of cancer* 49, 3924–3935 (2013).
21. Josse, C. et al. Identification of a microRNA landscape targeting the PI3K/Akt signaling pathway in inflammation-induced colorectal carcinogenesis. *Am J Physiol Gastrointest Liver Physiol* 306, G229–243 (2014).
22. Feng, Y. et al. A feedback circuit between mirt-133 and the ERK1/2 pathway involving an exquisite mechanism for regulating myoblast proliferation and differentiation. *Cell death & disease* 4, e934 (2013).
23. Li, Q., Lin, X., Yang, X. & Chang, J. NFA1c4 is negatively regulated in mirt-133a-mediated cardiomyocyte hypertrophic repression. *American journal of physiology. Heart and circulatory physiology* 298, H3140–H3147 (2010).
24. Dong, Y. et al. Tumor suppressor functions of mirt-133a in colorectal cancer. *Molecular cancer research: MCR* 11, 1051–1060 (2013).
25. Burman, J. L., Wasiak, S., Ritter, B., de Heuvel, E. & McPherson, P. S. Aftiphilin is a component of the clathrin machinery in neurons. *FEBS Lett* 579, 2177–2184 (2005).
26. Hirst, J., Borner, G. H., Harbour, M. & Robinson, M. S. The aftiphilin/p200/gamma-synergin complex. *Mol Biol Cell* 16, 2554–2565 (2005).
27. Lui-Roberts, W. W., Ferraro, F., Nightingale, T. D. & Cutler, D. F. Aftiphilin and gamma-synergin are required for secretagogue sensitivity of Weibel-Palade bodies in endothelial cells. *Mol Biol Cell* 19, 5072–5081 (2008).
28. Rao, P. K., Kumar, R. M., Farhkhondeh, M., Baskerville, S. & Lodish, H. F. Myogenic factors that regulate expression of muscle-specific microRNAs. *Proceedings of the National Academy of Sciences of the United States of America* 103, 8721–8726 (2006).
29. Chen, X. et al. In vitro evidence suggests that mirt-133a-mediated regulation of uncoupling protein 2 (UCP2) is an indispensable step in myogenic differentiation. *J Biol Chem* 284, 5362–5369 (2009).
30. Hua, Y., Zhang, Y. & Ren, J. IGF-1 deficiency resists cardiac hypertrophy and myocardial contractile dysfunction: role of microRNA-1 and microRNA-133a. *Journal of Cellular and Molecular Medicine* 16, 83–95 (2012).
31. Castoldi, G. et al. MicroRNA-133a regulates collagen 1A1: Potential role of microRNA-133a in myocardial fibrosis in angiotsenin II-dependent hypertension. *Journal of Cellular Physiology* 227, 850–856 (2012).
32. Roderburg, C. et al. miR-133a mediates TGF-beta-dependent derepression of collagen synthesis in hepatic stellate cells during liver fibrosis. *Journal of hepatology* 58, 736–742 (2013).
33. Arndt, G. M. et al. Characterization of global microRNA expression reveals oncogenic potential of miR-145 in metastatic colorectal cancer. *BMC cancer* 9, 374 (2009).
34. Sarver, A. L. et al. Human colon cancer profiles show differential microRNA expression depending on mismatch repair status and are characteristic of undifferentiated proliferative states. *BMC cancer* 9, 401 (2009).
35. Necela, B. M., Carr, J. M., Asmann, Y. W. & Thompson, E. A. Differential expression of microRNAs in tumors from chronically inflamed or genetic (APC(Min/+)) models of colon cancer. *PLoS One* 6, e18501 (2011).
36. Li, Z., Gu, X., Fang, Y., Xiang, J. & Chen, Z. microRNA expression profiles in human colorectal cancers with brain metastases. *Oncology letters* 3, 346–350 (2012).
37. Ma, Y. et al. Candidate microRNA biomarkers in human colorectal cancer: Systematic review profiling studies and experimental validation. *International Journal of Cancer* 130, 2077–2087 (2012).
38. Hermans, E., Geurts, N. M. & Maloteaux, J. M. Agonist and antagonist modulation of [35S]-GTP gamma S binding in transfected CHO cells expressing the neurotensin receptor. *Br J Pharmacol* 121, 1817–1823 (1997).
39. Yoshimori, T., Yamamoto, A., Moriyama, Y., Futai, M. & Tashiro, Y. Bafilomycin A1, a specific inhibitor of vacuolar-type ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J Biol Chem* 266, 17707–17712 (1991).
40. Demaurex, N., Furuya, W., D’Souza, S., Bonifacino, J. S. & Grinstein, S. Mechanism of Acidification of the trans-Golgi Network (TGN). *Journal of Biological Chemistry* 273, 2044–2051 (1998).
41. D’Souza, S. et al. The Epithelial Sodium-Hydrogen Antporter Na+/H+ Exchanger 3 Accumulates and Is Functional in Recycling Endosomes. *Journal of Biological Chemistry* 273, 2035–2043 (1998).
42. Teter, K. et al. Cellubrevin-targeted Fluorescence Uncovers Heterogeneity in the Recycling Endosomes. *Journal of Biological Chemistry* 273, 19625–19633 (1998).
43. Yamashiro, D. I. & Maxfield, F. R. Acidification of morphologically distinct endosomes in mutant and wild-type Chinese hamster ovary cells. *The Journal of Cell Biology* 105, 2723–2733 (1987).
44. Machen, T. et al. pH of TGN and recycling endosomes of H+/K+-ATPase-transfected HEK 293 cells: implications for pH regulation in the secretory pathway. *American journal of physiology. Cell physiology* 285, C205–C214 (2003).
45. Kozik, P. et al. A human genome-wide screen for regulators of clathrin-coated vesicle formation reveals an unexpected role for the V-ATPase. *Nat Cell Biol* 15, 50–53 (2013).
46. Wagner, M., Rajasekaran, A. K., Hanzel, D. K., Mayor, S. & Rodriguez-Boulan, E. Brefeldin A causes structural and functional alterations of the trans-Golgi network of MDCK cells. *J Cell Biol* 107 (Pt 4), 933–943 (1994).
47. Lippincott-Schwartz, J., Yuan, L. C., Bonifacino, J. S. & Klausner, R. D. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane recycling from Golgi to ER. *Cell* 56, 801–813 (1989).
48. Gómez-Benito, M. et al. EVI1 controls proliferation in acute myeloid leukemia through modulation of miR-1-2. *Br J Cancer* 103, 1292–1296 (2010).
49. Rajabi, H. et al. MUC1-C oncprotein activates the ZEB1/miR-200c regulatory loop and epithelial-mesenchymal transition. *Oncogene* 33, 1680–1689 (2014).
50. Bracken, C. P. et al. A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. *Cancer Res* 68, 7846–7854 (2008).
51. Liu, Y. et al. Zeb1 represses Mif1 and regulates pigment synthesis, cell proliferation, and epithelial morphology. *Invest Ophthalmol Vis Sci* 50, 5080–5088 (2009).
52. Toy-Miou-Leong, M., Cortes, C. L., Beaudet, A., Rostène, W. & Forgez, P. Receptor trafficking via the perinuclear recycling compartment accompanied by cell division is necessary for permanent neurotensin cell sensitization and leads to chronic mitogen-activated protein kinase activation. *J Biol Chem* 279, 12636–12646 (2004).
53. Lee, R. C., Feinbaum, R. L. & Ambros, V. The C. elegans heterochronic gene lin-4 mediates temporal complementarity to lin-14. *Cell* 75, 843–854 (1993).
54. Wightman, B., Ha, I. & Ruvkun, G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell* 75, 855–862 (1993).
55. Lim, L. P. et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 433, 769–773 (2005).
56. Johannes, L. & Popovic, V. Tracing the retrograde route in protein trafficking. *Cell* 135, 1175–1187 (2008).
57. Bonifacino, J. S. & Rojas, R. Retrograde transport from endosomes to the trans-Golgi network. *Nat Rev Mol Cell Biol* 7, 568–579 (2006).
58. Lu, L., Tai, G. & Hong, W. Autoantigen Golgin-97, an effector of Arf1 GTPase, participates in traffic from the endosome to the trans-Golgi network. *Mol Biol Cell* 15, 4426–4443 (2004).
59. Tai, G., Lu, L., Johannes, L. & Hong, W. Functional analysis of Arl1 and golgin-97 in endosome-to-TGN transport using recombinant Shiga toxin B fragment. *Methods in enzymology* **404**, 442–453 (2005).
60. Marquardt, D. & Center, M. S. Drug transport mechanisms in HL60 cells isolated for resistance to adriamycin: evidence for nuclear drug accumulation and redistribution in resistant cells. *Cancer Res* **52**, 3157–3163 (1992).
61. Naldini, L., Blömer, U., Gage, F. H., Trono, D. & Verma, I. M. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 11382–11388 (1996).
62. Turvy, D. N. & Blum, J. S. Biotin Labeling and Quantitation of Cell-Surface Proteins. In *Current Protocols in Immunology* (John Wiley & Sons, Inc., 2001).
63. Gardner, L. A., Hajjhussein, H., Frederick-Dyer, K. C. & Bahouth, S. W. Rab11a and its binding partners regulate the recycling of the sst1-adrenergic receptor. *Cellular signalling* **23**, 46–57 (2011).
64. Giles, D. K. & Wyrick, P. R. Trafficking of chlamydial antigens to the endoplasmic reticulum of infected epithelial cells. *Microbes and infection/Institut Pasteur* **10**, 1494–1503 (2008).

**Acknowledgements**
Supported by the National Institutes of Health DK60729, DK 47343 (CP), the Neuroendocrine Assay Core supported by P50 DK 64539 (CP), and a Research Fellowship from The Crohn’s and Colitis Foundation of America, Inc (IKML). Support was also provided by NHMRC 63303, 1049682, 1031886 and ARC (NWB.), the Blinder Research Foundation for Crohn’s Disease and the Eli and Edythe Broad Chair (CP). We would like to acknowledge the UCLA Vector Core and the Imaging and Stem Cell Biology Core (ISCB) supported by JCCC/P30 CA016042 and CURE/P30 DK041301 for the generation of recombinant lentivirus expressing NTR1 and AFTPH and use of confocal microscopy.

**Author Contributions**
I.K.M.L. and C.P. conceived and coordinated the study and wrote the paper. I.K.M.L. performed and analyzed the experiments. D.J. and N.W.B. provided technical assistance in confocal microscopy and contributed to the preparation of the Figures 2–6. All authors reviewed the results and approved the final version of the manuscript.

**Additional Information**
Supplementary information accompanies this paper at http://www.nature.com/srep

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Law, I. K. M. *et al.* Neurotensin-induced miR-133α expression regulates neurotensin receptor 1 recycling through its downstream target aftiphilin. *Sci. Rep.* **6**, 22195; doi: 10.1038/srep22195 (2016). This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/