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Corresponding Author:	Claus Munck Petersen Aarhus Universitet Aarhus, DENMARK
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	cmp@biomed.au.dk
Corresponding Author's Institution:	Aarhus Universitet
Corresponding Author's Secondary Institution:	
First Author:	Jakob Vejby Larsen
First Author Secondary Information:	
Other Authors:	Jakob Vejby Larsen
Order of Authors Secondary Information:	
Abstract:	The heterodimeric cytokine cardiotrophin-like cytokine:cytokine-like factor-1 (CLC:CLF-1) targets the glycosylphosphatidylinisotol (gpi)-anchored CNTFRα to form a trimeric complex that subsequently recruits glycoprotein 130/leukemia Inhibitory factor receptor-β (gp130/LIFRβ) for signaling. Both CLC and CNTFRα are necessary for signaling but so far CLF-1 has only been known as a putative facilitator of CLC secretion. However, it has recently been shown that CLF-1 contains three binding sites: one for CLC; one for CNTFRα (that may promote assembly of the trimeric complex); and one for the endocytic receptor sorLA. The latter site provides high affinity binding of CLF-1, CLC:CLF-1, as well as the trimeric (CLC:CLF-1:CNTFRα) complex to sorLA, and in sorLA-expressing cells the soluble ligands CLF-1 and CLC:CLF-1 are rapidly taken up and internalized. In cells co-expressing CNTFRα and sorLA, CNTFRα first binds CLC:CLF-1 to form a membrane associated trimeric complex, but it also connects to sorLA via the free sorLA-binding site in CLF-1. As a result CNTFRα, which has no capacity for endocytosis on its own, is tugged along and internalized by the sorLA-mediated endocytosis of CLC:CLF-1. The present protocol describes the experimental procedures used to demonstrate i) the sorLA-mediated and CLC:CLF-1-dependent downregulation of surface-membrane CNTFRα expression; ii) sorLA-mediated endocytosis and lysosomal targeting of CNTFRα; and iii) the lowered cellular response to CLC:CLF-1-stimulation upon sorLA-mediated downregulation of CNTFRα.
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TITLE:
SorLA and CLC:CLF-1-dependent downregulation of CNTFRα as demonstrated by Western blotting, inhibition of lysosomal enzymes, and immunocytochemistry

AUTHORS:
Larsen, Jakob V
Department of Biomedicine
Aarhus University
Aarhus, Denmark
jvl@biomed.au.dk

Petersen, Claus M
Department of Biomedicine
Aarhus University
Aarhus, Denmark
cmp@biomed.au.dk

CORRESPONDING AUTHOR:
Jakob Vejby Larsen
+45 87167821

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SHORT ABSTRACT:
The present protocol describes how Western blotting and immunocytochemistry combined with inhibition of lysosomal enzymes can be used to demonstrate the downregulation of ciliary neurotrophic factor receptor-α (CNTFRα) which is conveyed by the interaction between cytokine-like factor-1 (CLF-1) and the endocytic receptor sorLA.

LONG ABSTRACT:
The heterodimeric cytokine cardiotrophin-like cytokine:cytokine-like factor-1 (CLC:CLF-1) targets the glycosylphosphatidylinisotol (gpi)-anchored CNTFRα to form a trimeric complex that subsequently recruits glycoprotein 130/leukemia Inhibitory factor receptor-β (gp130/LIFRβ) for signaling. Both CLC and CNTFRα are necessary for signaling but so far CLF-1 has only been known as a putative facilitator of CLC secretion. However, it has recently been shown that CLF-1 contains three binding sites: one for CLC; one for CNTFRα (that may promote assembly of the trimeric complex); and one for the endocytic receptor sorLA. The latter site provides high affinity binding of CLF-1, CLC:CLF-1, as well as the trimeric (CLC:CLF-1:CNTFRα) complex to sorLA, and in sorLA-expressing cells the soluble ligands CLF-1 and CLC:CLF-1 are rapidly taken up and internalized. In cells co-expressing CNTFRα and sorLA, CNTFRα first binds CLC:CLF-1 to form a membrane associated trimeric complex, but it also connects to sorLA via the free sorLA-binding site in CLF-1. As a result CNTFRα, which has no capacity for endocytosis on its own, is

tugged along and internalized by the sorLA-mediated endocytosis of CLC:CLF-1.

The present protocol describes the experimental procedures used to demonstrate i) the sorLA-mediated and CLC:CLF-1-dependent downregulation of surface-membrane CNTFRα expression; ii) sorLA-mediated endocytosis and lysosomal targeting of CNTFRα; and iii) the lowered cellular response to CLC:CLF-1-stimulation upon sorLA-mediated downregulation of CNTFRα.

INTRODUCTION:

The CLC and the CLF-1 constitute the two subunits of the heterodimeric cytokine CLC:CLF-1[1-3]. For cellular signal induction CLC:CLF-1 first targets the CNTFRα, its primary and gpi-anchored receptor, to form a membrane-bound trimeric complex. The CLC:CLF-1:CNTFRα complex subsequently recruits gp130/LIFRβ which mediates transmembrane signaling via the Janus kinase/signal transducers and activators of transcription 3 (JAK/STAT3) pathway[4]. Mice deficient in any of the three subunits display one and the same phenotype and die within 24 h after birth due to a reduced number of facial neurons and insufficient suckling[5-8]. Findings in humans likewise underscore the functional coherence of the three subunits. Thus, human mutations (homozygote or compound) causing dysfunction of CLC:CLF-1 or CNTFRα all result in the so-called cold-induced sweating/Crisponi syndrome, a condition characterized by e.g. impaired suckling and swallowing, various dysmorphic features, temperature spikes, and paradoxical and perfuse sweating at low temperatures[9-11].

In vitro the combination of CLC and CNTFRα is both necessary and sufficient for interaction with gp130/LIFRβ and the induction of signaling in cells[12]. The role of CLF-1 on the other hand is less clear. It is not directly involved in signaling, and has long been regarded as an appendix that mainly serves to facilitate the cellular secretion of CLC[1]. However, recent findings show that CLF-1 has additional and more important functions implicating both the signaling and turnover of CLC and CNTFRα. Thus, it appears that CLF-1 contains three independent binding sites: one for its well-known binding to CLC; one that mediates direct binding to the CNTFRα; and a third (high affinity) site for interaction with the endocytic receptor sorLA. As both CLC and CLF-1 seems to target CNTFRα with a considerable lower affinity than the CLC:CLF-1 complex, it is conceivable that CLF-1 (via its CNTFRα-binding site) promotes the unification of CLC and CNTFRα and thereby facilitates signaling[13].

CLF-1's interaction with sorLA, the main issue of the present presentation, plays a completely different role. SorLA is one of the five type 1 receptors that constitute the Vps10p-domain receptor family[14]. It is expressed in a variety of tissues but in particular in brain and neuronal tissues[15]. Similar to the other family members sorLA carries an N-terminal ligand binding Vps10p-domain, but in addition it also comprises other domain types including ligand-binding elements found in members of the low-density lipoprotein receptor family[15]. Its cytoplasmic tail interacts with several adaptor proteins, e.g. adaptor protein-1 and -2, and sorLA conveys efficient endocytosis as well as intracellular sorting and transport of bound proteins[16-18]. The Vps10p-domain comprises a large ten-bladed β-propeller[19,20], which binds a series of unrelated ligands including CLF-1 (but notably, not CLC)[13]. The binding site in CLF-1 is accessible even after

complex formation with CLC and CNTFRα, which means that sorLA binds not only free CLF-1, but also CLC:CLF-1 and the trimeric complex CLC:CLF-1:CNTFRα[13]. SorLA conveys rapid endocytosis of CLF-1 and CLC:CLF-1, but these are soluble proteins whereas CNTFRα is fixed to the surface membrane by a gpi-anchor. The question is therefore if binding of CLC:CLF-1:CNTFRα allows sorLA to internalize the entire complex and thereby to alter the surface-membrane expression of CNTFRα (and the subsequent cellular susceptibility to CLC:CLF-1 signal induction), and/or the turnover of CNTFRα.

The experiments described in the present report were designed to clarify the following questions.

Does sorLA mediate CLC:CLF-1-dependent downregulation of surface-membrane CNTFRα? To clarify this, it was initially tried to determine the turnover of CNTFRα (in the absence and presence of sorLA) by means of metabolic labeling and pulse-chase experiments as described in[21]. However, attempts to label CNTFRα using a mixture of [S^{35}] cysteine and [S^{35}] methionine showed poor incorporation of radioactivity. This suggested a very low degree of new-synthesis, which in all probability would be unable to compensate for a sudden and significant loss of receptors. To determine if CNTFRα was downregulated when interconnected via CLC:CLF-1 to sorLA, it was therefore decided simply to measure (using Western blotting) the total cellular pool of CNTFRα before and after exposure to CLC:CLF-1 - and to compare results in cells transfected or not transfected with sorLA.

Does sorLA target CNTFRα to lysosomes? To answer this question, the localization of CNTFRα - internalized via its CLC:CLF-1-mediated binding to sorLA - was examined using immunocytochemistry, and labeling with antibodies directed towards CNTFRα and the late-endosome/lysosome marker lysosomal-associated membrane protein 1 (LAMP-1). The experiment was performed on cells treated as well as untreated with inhibitors of lysosomal enzymes. The idea was of course that if CNTFRα was degraded in lysosomes, cells treated with enzyme-inhibitors would present CNTFRα-staining accumulating in LAMP-1 positive vesicles, whereas untreated cells would show little or no staining for CNTFRα.

Does CNTFRα downregulation lower the cellular response to CLC:CLF-1 stimulation? The trimeric complex consisting of CLC, CLF-1, and CNTFRα signals via the gp130/LIFRβ heterodimer using the JAK/STAT3 pathway. Accordingly, the capacity of cells to respond to CLC:CLF-1 signaling upon downregulation of CNTFRα was explored by Western blot detection and quantitation of the phospho-STAT3 (pSTAT3)/total STAT3 level in sorLA transfectants.

PROTOCOL:

1. Western blotting of CNTFRα lysates

1.1. Express full-length sorLA and Myc-tagged CNTFRα constructs in human embryonic kidney 293 (HEK293) cells using pcDNA3.1/zeo(-) and pcDNA3.1/hyg(-), respectively. Transfect the HEK293 cells with the constructs using a commercial transfection reagent according to

manufacturer´s protocol. Select stable clones in medium containing 150 μg/mL Zeocin and/or 500 μg/mL Hygromycin B Gold[13].

1.2. Seed equal numbers of HEK293 transfectants expressing either CNTFRα-Myc or co-expressing CNTFRα-Myc/sorLA in two 4-well plates as shown in Figure 1. Culture the cells in Dulbecco Modified Eagle´s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (full medium) in a 5% carbon dioxide incubator at 37 ˚C.

1.3. Wait until the cells are 50-80% confluent.

1.4. Remove the medium from the wells, wash once with Dulbecco Phosphate Buffered Saline (PBS), and add fresh full medium with or without (control) 10 nM CLC:CLF-1 as indicated in Figure 1. Incubate the cells at 37 ˚C.

1.5. After 0 and 5 h of incubation, recover the medium and store it at 4 ˚C in 1.5 mL tubes. Then add 1% Triton X-100 lysis buffer (20 mM Tris-HCl, 10 mM EDTA, pH 8.0) supplemented with a proteinase inhibitor cocktail to each well (1 min, 4 ˚C). Subsequently, transfer the cell lysates to 1.5 mL tubes (4 ˚C).

1.6. Spin the tubes containing the medium and cell lysate (3000 x g, 3 min, 4 ˚C) and transfer the supernatants to 1.5 mL tubes (4 ˚C). Discard the pellet. Transfer 5 μL of each cell lysate supernatant to new 1.5 mL tubes - use them to measure the protein content later (step 1.6). Immediately, add non-reducing LDS sample buffer to all the supernatants and vortex the samples.

1.7. Use the 5 μL aliquots to measure the protein content in the cell lysate supernatants using a commercial protein assay according to manufacturer's protocol.

1.8. Heat the samples at 95 ˚C for 5 min.

1.9. Subject an equal amount of protein from each sample to reducing SDS-PAGE[13] and Western blotting[13] and visualize the content of sorLA, β-actin, and CNTFRα-Myc in the medium and in the cell lysates using rabbit anti-sorLA[16] (5 μg/mL), mouse anti-β-actin (0.4 μg/mL), and mouse anti-Myc (1 μg/mL) antibodies and the appropriate HRP-linked secondary antibodies (1:2000).

1.10. Quantify the bands by densitometry according to manufacturer's protocol.

2. Immunocytochemistry in combination with lysosomal inhibitors

2.1) Seed HEK293 cells stably transfected with CNTFRα-Myc/sorLA on cover slides in a 4-well plate as shown in Figure 2. Culture the cells in full medium as above.

2.2) Wait until cell confluence is 20-40%.

2.3) Remove the medium from the wells and add full medium with or without 50 μg/mL leupeptin and pepstatin A (leu/pep) (inhibits lysosomal hydrolases) to the cells as indicated in Figure 2. Incubate the cells at 37 ˚C. The medium must be replaced every 6 h during the following 24 h of incubation.

2.4) After 24 h of incubation, once again replace the medium (with or without leu/pep) and this time add 10 nM CLC:CLF-1. Incubate the cells at 37 ˚C for 5 h.

2.5) Wash the cells once in PBS and fix them in 4% paraformaldehyde, pH 7 for 15 min at room temperature.

Caution: Paraformaldehyde is highly toxic, avoid contact with skin, eyes, and mucous. Gloves and safety glasses should be worn and solutions made inside a fume hood).

2.6) Wash the cells once in PBS and permeabilize the cells for 5 min at room temperature in saponin diluted in PBS (0.5% saponin).

2.7) Dilute goat anti-CNTFRα (1 μg/mL) (notably not the mouse anti-Myc used for Western blotting) and mouse anti-LAMP-1 (15 μg/mL) antibodies in 0.5% saponin and incubate the cells with the antibodies for 2 h at room temperature.

2.8) Wash the cells 4 times (5 min) in 0.5% saponin and incubate the cells with donkey anti-goat Alexa 488- and donkey anti-mouse Alexa 568-conjugated secondary antibodies (6 μg/mL) for 2 h at room temperature.

2.9) Wash the cells 4 times (5 min) in 0.5% saponin, 2 times in deionized water (1 min), and mount the cover slides on microscope slides using mounting media.

2.10) Analyze the antibody-staining of the cells by confocal microscopy using a laser-scanning confocal microscope with a 63 X C-apochromat water immersion objective, NA 1.2.

3. Western blot-detection of the pSTAT3 level

3.1) Seed HEK293-sorLA cells (which express endogenous levels of CNTFRα) in a 4-well plate as shown in Figure 3. Culture the cells in full medium as above.

3.2) Wait until the cells are 50-80% confluent.

3.3) Remove the medium from the wells, wash once in PBS, and add fresh full medium with or without 10 nM CLC:CLF-1 to the cells as indicated in Figure 3. Incubate the cells at 37 ˚C for 5 h.

3.4) Remove the medium, wash the cells twice in unsupplemented DMEM (blank medium;

without FBS and antibiotics), and then re-incubate the cells for 90 min (37 ˚C) in blank medium.

3.5) Once again, remove the medium and add fresh blank medium with or without 5 nM CLC:CLF-1 (to initiate STAT3 phosphorylation) as indicated in figure 3. Incubate for 15 min at 37 ˚C.

3.6) Quickly remove the medium and lyse the cells (1 min, 4 ˚C) by adding 1% Triton X-100 lysis buffer (20 mM Tris-HCl, 10 mM EDTA, pH 8.0) supplemented with a proteinase inhibitor cocktail and a phosphatase inhibitor cocktail to each well. Subsequently, transfer the cell lysates to 1.5 mL tubes (4 ˚C).

3.7) Transfer 5 μl of each supernatant to new 1.5 mL tubes - use them to measure the protein content later (step 3.7). Immediately, add non-reducing LDS sample buffer to all the supernatants and vortex the samples.

3.8) Measure the protein content of the 5 μL aliquots using a commercial protein assay according to manufacturer's protocol.

3.9) Sonicate each sample at room temperature in a cup horn with an output level of 95% and a constant duty cycle until (approximately 1 min) they are no longer viscous and subsequently heat them at 95 ˚C for 5 min.

3.10) Subject an equal amount of protein from each sample to SDS-PAGE[13] and Western blotting[13] and visualize the content of pSTAT3 and total STAT3 in the cell lysates using rabbit anti-pSTAT3 (1:2000) and mouse anti-STAT3 (1:1000) antibodies and the appropriate HRP-linked mouse and rabbit secondary antibodies (1:2000).

3.11) Quantify the bands by densitometry according to manufacturer's protocol.

REPRESENTATIVE RESULTS:

Figure 1 shows a schematic representation of the cell-seeding and CLC:CLF-1 incubation in protocol 1. **Figure 2** shows a schematic representation of the cell-seeding and leu/pep and CLC:CLF-1 incubation in protocol 2. **Figure 3** shows a schematic representation of the cell-seeding and CLC:CLF-1 incubation and stimulation in protocol 3. **Figure 4** shows the sorLA-mediated CLC:CLF-1-dependent downregulation of CNTFRα. **Figure 5** shows the sorLA-mediated endocytosis and lysosomal targeting of CNTFRα. **Figure 6** shows the lower response to CLC:CLF-1 stimulation after CNTFRα downregulation.

Note that bands signifying CNTFRα-Myc have similar density at time zero, whereas downregulation of CNTFRα-Myc is demonstrated by the weaker band in sorLA transfectants after 5 h (Figure 4). Figure 5 shows that CNTFRα-Myc has accumulated in LAMP-1 positive vesicles of leu/pep treated cells. In contrast, no accumulation of CNTFRα-Myc is seen in cells not subjected to leu/pep treatment. This demonstrates that CNTFRα-Myc is sorted to

lysosomes and degraded. As can be seen in Figure 6, the level of pSTAT3 is significantly reduced in pre-stimulated cells as compared to the level in cells that has not been exposed to CLC:CLF-1. This is in accordance with the observed sorLA-mediated downregulation of CNTFRα in the pre-stimulated cells (Figure 4).

Figure 1: Schematic representation of cell-seeding and CLC:CLF-1 incubation in protocol 1.
Seed HEK293-CNTFRα-Myc and HEK293-CNTFRα-Myc/sorLA cells in two 4-well plates (0 and 5 h) and incubate cells with CLC:CLF-1 as indicated.

Figure 2: Schematic representation of cell-seeding and leu/pep and CLC:CLF-1 incubation in protocol 2.
Seed HEK293-CNTFRα-Myc/sorLA cells in one 4-well plate and incubate cells with or without leu/pep and CLC:CLF-1 as indicated.

Figure 3: Schematic representation of cell-seeding and CLC:CLF incubation and stimulation in protocol 3.
Seed HEK293-sorLA cells in one 4-well plate and incubate cells with or without CLC:CLF-1 (pre-exp.) to downregulate the CNTFRα followed by CLC:CLF-1 stimulation (stim.) to initiate STAT3 phosphorylation as indicated.

Figure 4: Results showing that sorLA mediates CLC:CLF-1-dependent downregulation of CNTFRα.
(A) HEK293-CNTFRα-Myc and (B) HEK293-CNTFRα-Myc/sorLA cells were incubated in the absence (white columns) or presence (grey columns) of 10 nM CLC:CLF-1. After 0 and 5 h, the incubation was stopped and the content of CNTFRα-Myc in the medium (m) and in the cell lysates (l) was detected by Western blotting and quantified by densitometry. The upper panels show Western blot results from representative experiments and the lower panels show the detected levels of CNTFRα-Myc found in the cell lysates. The levels are shown relative to the CNTFRα-Myc level in the single and double transfectants at 0 h. Each column represents mean ± SEM (n=3). p-values calculated using t-test.
Reproduced after original figure[13]

Figure 5: Results showing that sorLA mediate endocytosis and lysosomal targeting of CNTFRα.
HEK293-CNTFRα-Myc/sorLA cells were treated with or without leu/pep, incubated with 10 nM CLC:CLF-1 for 5 h, fixed, and finally stained using anti-CNTFRα and anti-LAMP-1 antibodies as described in protocol 2. Scale bars: 5 μm.
Reproduced after original figure[13].

Figure 6: SorLA-mediated downregulation of CNTFRα is accompanied by a lowered cellular response to CLC:CLF-1 stimulation.
HEK293-sorLA cells were pre-incubated in the absence or presence of 10 nM CLC:CLF-1 for 5 h (pre-exp.), starved in blank medium for 90 min, and stimulated with 5 nM CLC:CLF-1 for 15 min (stim.). (A) The columns show the relative levels of pSTAT3 in the cells. Each column represents

mean ± SEM (n=3) relative to the pSTAT3 level in cells pre-incubated in the absence of CLC:CLF-1 but stimulated with CLC:CLF-1. p-value calculated using t-test. (B) The Western blot shows the response of pSTAT3 and STAT3 obtained in a representative experiment.
Reproduced after original figure[13].

DISCUSSION:
The protocol described here can be used specifically to demonstrate the sorLA-mediated CLC:CLF-1-dependent downregulation and lysosomal targeting of CNTFRα, as well as the accompanying weakened response to CLC:CLF-1 stimulation.

The HEK293 cell line is well-suited for this protocol, as they have only a minor endogenous expression of CNTFRα and sorLA, are easy to transfect, express gp130/LIFRβ, and have a large cell body, which is suited for immunocytochemistry. However, in theory any cell line with similar properties should work as well.

The protocol has two critical steps. The first concerns step 2.3, in which the leu/pep medium must be replaced approximately every 6 h for 24 h. Failure to do so may result in active lysosomal enzymes and less or no detectable accumulation of protein in the lysosomes. The second critical step (3.4) concerns washing upon pre-incubation with CLC:CLF-1. It is important to remove any unbound ligand, and to allow the cells time to recover (avoiding continued stimulation) in unsupplemented DMEM (blank medium). This will ensure a low background level of pSTAT3 during the subsequent re-stimulation with CLC:CLF-1 (step 3.5).

Notably, Figure 6 demonstrates that the cellular response (pSTAT3) decreases in parallel with the sorLA-mediated downregulation of CNTFRα. It should be emphasized however, that although a reduced response is in accordance with (and to be expected upon) a reduction of the CNTFRα pool, it does not prove that the low CNTFRα expression alone accounts for the reduced cellular response. Thus, changes - resulting from pre-stimulation or transfection - in any of the functional elements of the signaling pathway upstream to pSTAT3 may have contributed to the altered response.

The basic concept of the protocol is not limited to this particular receptor system. By modifying the protocol (i.e. another cell line, other antibodies, other ligands etc.) it can be used to determine the ligand-induced downregulation of other receptors as well - regardless of sorLA´s involvement. However, it should be noted that analysis of receptor-downregulation by Western blotting is mainly suited for receptors, e.g. gpi-anchored receptors, which exhibit a slow turnover and a low degree of new synthesis in unstimulated cells. Thus, in receptors showing a high level of new-synthesis, ligand-induced downregulation may be compensated for by synthesis of new receptors. Under these circumstances, the downregulation of the receptor pool can instead be determined by means of metabolic labeling and pulse-chase experiments as described in[21]. Alternatively, iodinated antibodies (preferably Fc-fragments) that do not interfere with receptor binding can be used to “tag” the ectodomain of the receptor, and the expected downregulation can then be determined by radioactive counting of cell pellet and medium.

For logistic reasons we transfected our cells with a CNTFRα construct carrying a Myc-tag, and in the present protocol a mouse anti-Myc antibody was used for Western blot-detection of the receptor. In contrast, a goat anti-CNTFRα antibody was used for immunocytochemistry and double labeling as LAMP-1 was detected by a mouse antibody - and obviously use of two primary mouse antibodies would result in unspecific (overlapping) staining with secondary antibodies. Note that since the goat anti-CNTFRα also works in Western blotting (not shown), the protocol could easily be modified and applied to cells transfected with untagged CNTFRα.

Finally, it has recently been shown that also the endocytic receptor sortilin binds CLC:CLF-1 with high affinity[22]. Thus, it is conceivable that sortilin, like sorLA, interconnects to CLC:CNTFRα via CLF-1 and is able to mediate endocytosis and lysosomal targeting of CNTFRα as well. Using sortilin instead of sorLA, the present protocol may be used to clarify this question.

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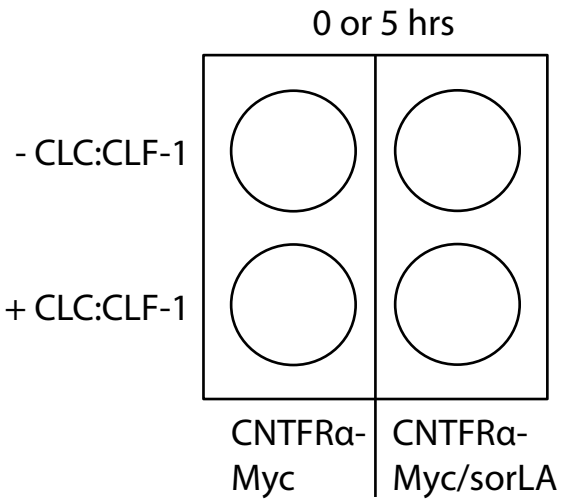
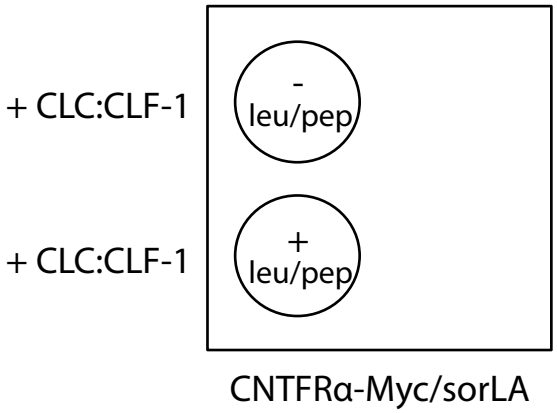
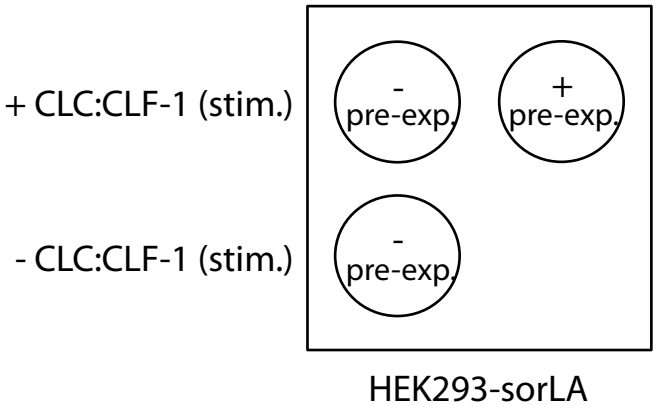
The authors have nothing to disclose.

REFERENCES:
1 Elson, G. C. *et al.* CLF associates with CLC to form a functional heteromeric ligand for the CNTF receptor complex. *Nat Neurosci.* **3** (9), 867-872 (2000).
2 Senaldi, G. *et al.* Novel neurotrophin-1/B cell-stimulating factor-3: a cytokine of the IL-6 family. *Proc Natl Acad Sci U S A.* **96** (20), 11458-11463 (1999).
3 Elson, G. C. *et al.* Cytokine-like factor-1, a novel soluble protein, shares homology with members of the cytokine type I receptor family. *J Immunol.* **161** (3), 1371-1379 (1998).
4 Lelievre, E. *et al.* Signaling pathways recruited by the cardiotrophin-like cytokine/cytokine-like factor-1 composite cytokine: specific requirement of the membrane-bound form of ciliary neurotrophic factor receptor alpha component. *J Biol Chem.* **276** (25), 22476-22484 (2001).
5 Zou, X. *et al.* Neonatal Death in Mice Lacking Cardiotrophin-Like Cytokine (CLC) is Associated with Multifocal Neuronal Hypoplasia. *Vet Pathol.* **46** (3), 514-519 (2008).
6 Forger, N. G. *et al.* Cardiotrophin-like cytokine/cytokine-like factor 1 is an essential trophic factor for lumbar and facial motoneurons in vivo. *J Neurosci.* **23** (26), 8854-8858 (2003).
7 Alexander, W. S. *et al.* Suckling defect in mice lacking the soluble haemopoietin receptor NR6. *Curr Biol.* **9** (11), 605-608 (1999).
8 DeChiara, T. M. *et al.* Mice lacking the CNTF receptor, unlike mice lacking CNTF, exhibit profound motor neuron deficits at birth. *Cell.* **83** (2), 313-322 (1995).
9 Crisponi, L. *et al.* Crisponi syndrome is caused by mutations in the CRLF1 gene and is allelic to cold-induced sweating syndrome type 1. *Am J Hum Genet.* **80** (5), 971-981, doi:10.1086/516843 (2007).
10 Crisponi, G. Autosomal recessive disorder with muscle contractions resembling neonatal

tetanus, characteristic face, camptodactyly, hyperthermia, and sudden death: a new syndrome? *Am J Med Genet.* **62** (4), 365-371, doi:10.1002/(SICI)1096-8628(19960424)62:4<365::AID-AJMG8>3.0.CO;2-Q (1996).
11 Sohar, E., Shoenfeld, Y., Udassin, R., Magazanik, A. & Revach, M. Cold-induced profuse sweating on back and chest. A new genetic entity? *Lancet.* **2** (8099), 1073-1074 (1978).
12 Guillet, C. *et al.* Functionally active fusion protein of the novel composite cytokine CLC/soluble CNTF receptor. *Eur J Biochem.* **269** (7), 1932-1941 (2002).
13 Larsen, J. V. *et al.* Cytokine-Like Factor 1, an Essential Facilitator of Cardiotrophin-Like Cytokine:Ciliary Neurotrophic Factor Receptor alpha Signaling and sorLA-Mediated Turnover. *Mol Cell Biol.* **36** (8), 1272-1286, doi:10.1128/MCB.00917-15 (2016).
14 Willnow, T. E., Petersen, C. M. & Nykjaer, A. VPS10P-domain receptors - regulators of neuronal viability and function. *Nat Rev Neurosci.* **9** (12), 899-909 (2008).
15 Jacobsen, L. *et al.* Molecular characterization of a novel human hybrid-type receptor that binds the alpha2-macroglobulin receptor-associated protein. *J Biol Chem.* **271** (49), 31379-31383 (1996).
16 Nielsen, M. S. *et al.* Sorting by the cytoplasmic domain of the amyloid precursor protein binding receptor SorLA. *Mol Cell Biol.* **27** (19), 6842-6851 (2007).
17 Jacobsen, L. *et al.* The sorLA cytoplasmic domain interacts with GGA1 and -2 and defines minimum requirements for GGA binding. *FEBS Lett.* **511** (1-3), 155-158 (2002).
18 Klinger, S. C. *et al.* SorLA regulates the activity of lipoprotein lipase by intracellular trafficking. *J Cell Sci.* **124** (Pt 7), 1095-1105, doi:10.1242/jcs.072538 (2011).
19 Kitago, Y. *et al.* Structural basis for amyloidogenic peptide recognition by sorLA. *Nat Struct Mol Biol.* **22** (3), 199-206, doi:10.1038/nsmb.2954 (2015).
20 Quistgaard, E. M. *et al.* Ligands bind to Sortilin in the tunnel of a ten-bladed beta-propeller domain. *Nat Struct Mol Biol.* **16** (1), 96-98 (2009).
21 Larsen, J. V. *et al.* Human sorCS1 binds sortilin and hampers its cellular functions. *Biochem J.* **457** (2), 277-288, doi:10.1042/BJ20130386 (2014).
22 Larsen, J. V. *et al.* Sortilin facilitates signaling of ciliary neurotrophic factor and related helical type 1 cytokines targeting the gp130/leukemia inhibitory factor receptor beta heterodimer. *Mol Cell Biol.* **30** (17), 4175-4187, doi:10.1128/MCB.00274-10 (2010).







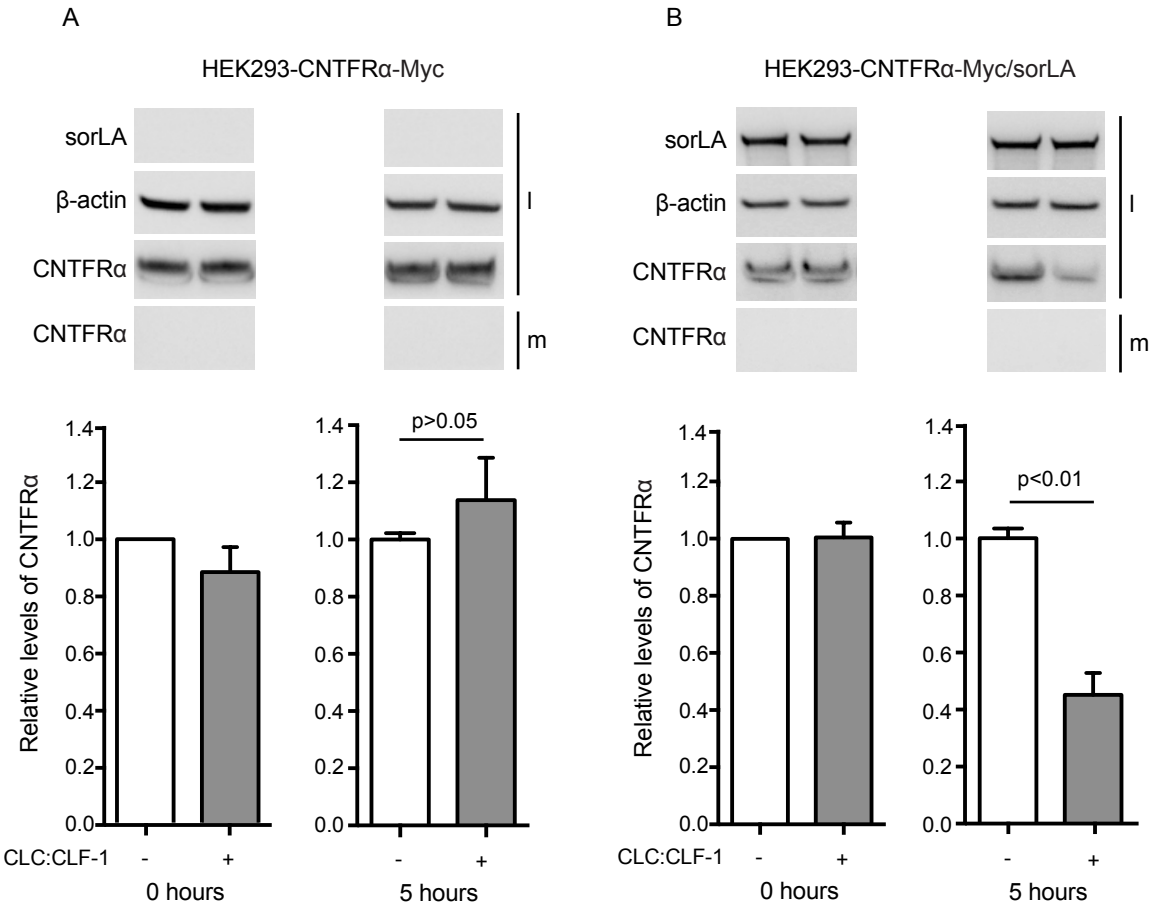
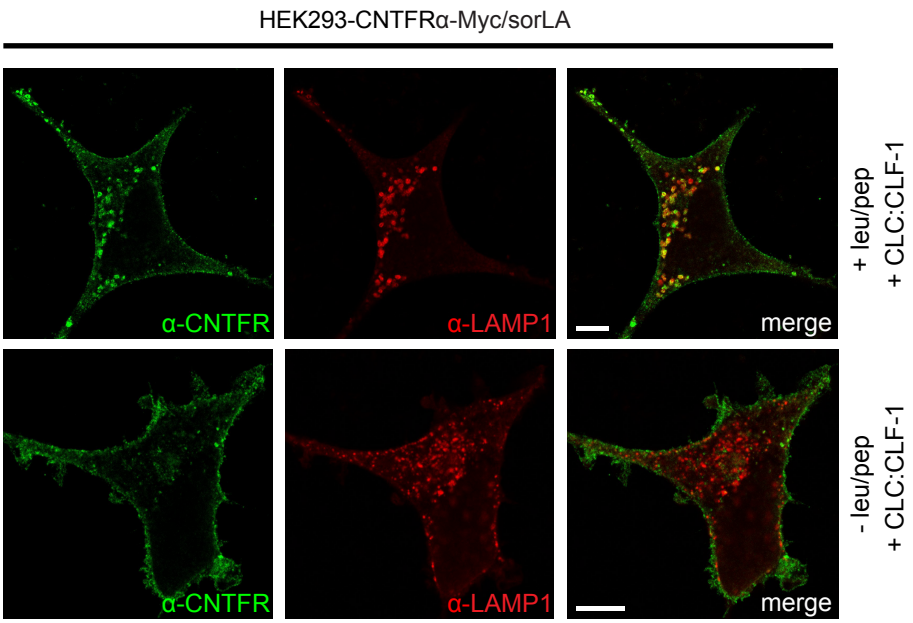
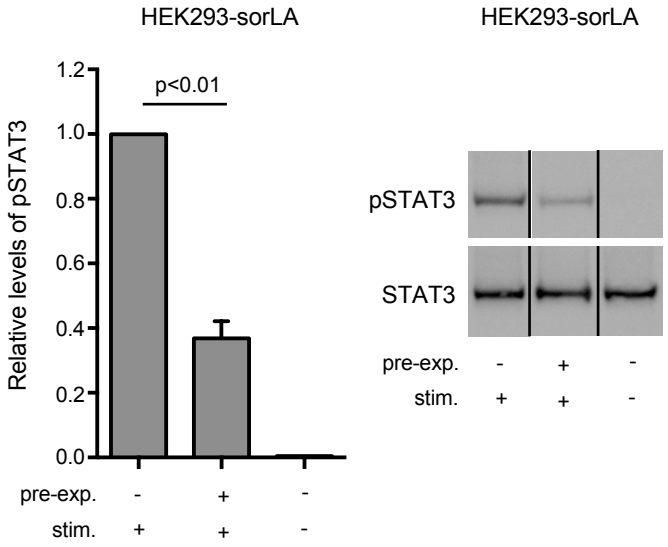


Figure 5





Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Zeocin	InvivoGen	ant-zn-1	
Hygromycin B Gold	InvivoGen	ant-hg-1	
FuGENE 6 Transfection Reagent	Roche	11 815 091 001	
4-well plates (Nunclon Delta Surface)	Thermo Scientific	176740	
Safe-Lock tubes	Eppendorf	0030 120.086	
Dulbecco Modified Eagle´s Medium	Lonza	BE12-604F/U1	
Fetal bovine serum	Thermo Scientific	10270106	
Penicillin-Streptomycin	Thermo Scientific	15140122	
Dulbecco Phosphate Buffered Saline	HyClone	SH30028.02	
CLC:CLF-1	R&D Systems	1151-CL/CF	
Mouse anti-LAMP-1	DSHP	H4A3	
Rabbit anti-sorLA			Reference 16
Mouse anti-b-actin	Sigma	A2228	
Mouse anti-Myc	Genscript	A00704	
Goat anti-CNTFRa	R&D Systems	AF-303	
Rabbit anti-pSTAT3	Cell signaling Technology	9145s	
Mouse anti-STAT3	Cell signaling Technology	9139s	
Anti-mouse HRP-linked antibody	Cell signaling Technology	7076s	
Anti-rabbit HRP-linked antibody	Cell signaling Technology	7074s	
Anti-goat HRP-linked antibody	Dako	P0160	
Donkey anti-goat secondary antibody, Alexa Fluor 488 conjugate	Invitrogen	A11055	
Donkey anti-mouse secondary antibody, Alexa Fluor 568 conjug	Invitrogen	A10037	
Leupeptin	Sigma	L8511-5MG	
Pepstatin A	Sigma	P4265-5MG	
Triton X-100	AppliChem	A1388	
Tris-HCl	Merck	1,082,191,000	
EDTA:Titriplex III	Merck	1,084,180,250	
cOmplete mini EDTA-free	Roche	11836170001	
PhosSTOP	Roche	04 906 837 001	

LDS sample Buffer (4X)	Novex	NP0007
Bio-Rad Protein Assay	Bio-Rad	500-0006
4% paraformaldehyde	VWR	97,135,000
Saponin	Sigma	S7900-100G
Mounting Media	Dako	S3023

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

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Author(s): Jakob Vejby Larsen, Claus Munck Petersen

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CORRESPONDING AUTHOR:

Name:	Jakob Vejby Larsen
Department:	Department of Biomedicin
Institution:	Aarhus University
Article Title:	SorLA mediates CLF-1-dependent endocytosis and downregulation of CNTFRα
Signature:	Jakob V Larsen
Date:	20.5.2016

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Editorial comments:

1. Formatting:

-Please change the title to reflect the method rather than an experimental result.

Done.

-Please re-write the short abstract to address the methods presented rather than the results.

Done.

-Please use italics for Latin phrases such as “in vivo” and “in vitro”.

Done.

-Paraformaldehyde is toxic and requires a caution statement.

Done.

-Please reformat bold protocol section headings to reflect the method rather than the result.

Done.

2. Grammar:

-Line 47 – The following what? Manuscript? Article?

The text has been changed (new manuscript, line 46).

-2.10 – “Analyze the stainings”

The text has been changed (new manuscript, line 199).

-Discussion - Please correct the punctuation in the third paragraph. A list should not be separated by complete sentences.

The text has been changed (new manuscript, lines 312-318).

-Line 306 – “for receptors which, when unstimulated, exhibits”

The text has been changed (new manuscript, lines 330-332).

3. Additional detail is required:

-1.2, 2.2, 3.2 – About how long does this take?

As long as it takes (it all depends on the initial cell-seeding density).

-2.7, 2.8 – Is this 5% saponin?

No it is 0.5% Saponin. 0.5% has been added to the text (new manuscript, lines 186, 189, 192, 196).

-2.8 – What antibody dilution?

Antibody dilutions have been added throughout the text.

-3.8 – What are the settings? Is this performed on ice?

The proper settings and conditions are now given (new manuscript, lines 230-232).

4. Branding:

-1.4, 3.5 – cOmplete

Done

-3.5 – PhosSTOP

Done

5. Results:

-Please describe the results (ie what the data show) in more detail in there results section. One sentence for figures 4, 5 and 6 is insufficient.

The results have been rewritten in accordance with the requests

-Please indicate any statistical tests used.

Done.

-Figure 5 requires a scale bar.

Done

Reviewer #1:
Manuscript Summary:
Comments for author

Overall an interesting paper but given that it is a methods paper one would expect at least as much methods detail as a standard paper. I would suggest a general increase in such detail. Specific points below.

Since it is a methods paper it would be best to not refer to the authors' standard (not methods) paper for details of the methods e.g., (lines 128, 197). It would be better to include the details in this current paper. For example, what are the specifics of the vectors. What procedure was used for transfection?

Information regarding constructs and reagents used for transfection are now included (new manuscript, lines 126-130).

Line 129 indicates myc tag not required but myc antibody is specified on line 157.

The line (line 129 in the original manus) has been omitted as it is somewhat confusing – instead, the use of a Myc-tag and anti-Myc Ab's has been clarified in the discussion section (new manuscript, lines 341-347).

Shouldn't the text on protocol points 1., 2., etc be the questions addressed not the conclusions drawn from the experiments.

The protocol points 1, 2, and 3 has been rewritten in accordance with the requests.

Why different cell confluence used in 1.2 vs 2.2 steps?

High confluency was used simply to ensure enough protein for Western blotting, whereas a lower confluency was preferred for immunocytochemistry to allow a clear view of single cells by avoiding overlap and clustering.

Specify all primary and secondary antibody concentrations used? This is generally included even in non-methods papers because it is so critical.

Antibody dilutions have been added throughout the text.

Saponin concentration used in antibody solution?

The Saponin concentration has been added to the text (new manuscript, lines 186, 189, 192, 196).

How do you know it is the downregulation of CNTFR that lowers the response to subsequent CLC:CLF-1 stimulation. All that is shown is that CLC/CLF-1 treatment lowers the response to subsequent treatment with the same ligand, a very common pharmacological effect that could be mediated by many different things independent of CNTFR. The authors need to discuss and qualify their conclusions and/or explain the logic.

The low response to CLC:CLF-1 in pre-stimulated cells is shown because it agrees with (and is to be expected upon) a downregulation of CNTFRα. But we fully agree with the reviewer and acknowledge that other factors may contribute - this has now been emphasized in the discussion section (new manuscript, lines 319-325).

Is there a spin step between steps 3.5 and 3.6?

No.

Typo line 282 ..figur
Corrected.

Typo line 309 in stead

Corrected

Line 306 exhibit not exhibits

corrected

Major Concerns: N/A

Minor Concerns: N/A

Additional Comments to Authors: N/A

Reviewer #2:
Manuscript Summary:
This manuscript summarises the techniques used by this group to investigate the role of CRLF1 in the functions of the cytokine CLCF1. It definitively complementary to the previous publications of this group and deserves to be published.

Major Concerns: N/A

Minor Concerns: Figure 6 is unclear. Are the cells used HEK293-CNTFRa-Myc/sorLA or HEK293-sorLA?

The cells used are HEK293-sorLA. As mentioned (new manuscript, line 203) they express endogenous CNTFRα (without a Myc-tag).

Additional Comments to Authors: N/A