1 # Effect of tolytoxin on tunneling nanotube formation and function
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15 **Supplementary material**
16 **Methods**
17 **Co-Culture Experiments**
18 *Co-culture preparation for DiD vesicle transfer experiments*
19 SW13 cells (donor) were plated on 35 mm dishes (Falcon) and treated with 3 nM tolytoxin or
20 3 nM methanol for 18 h in parallel to non-treated control cells. Then they were incubated with
21 vybrant DiD cell labeling solution (Thermo Fisher Scientific) (1:2000 in complete medium) for
22 45 min at 37 °C to label vesicle membranes. Cells were washed three times with 0.01% trypsin
23 to remove unbound dye. In parallel, SW13 cells (acceptor) were plated on T25 flasks and
24 transfected with 3 μg of H2B-mCherry plasmid. Donor and acceptor cells were trypsinized,
25 centrifuged (1000 rpm, 4 min), counted and co-cultured on 35 mm Ibidi μ-dishes for 24 h with
26 1:1 ratio (120.000 donor-120.000 acceptor). Same ratio was used for all co-culture preparations
27 in SW13 cells (Supplementary Fig. S4a). Cells were then fixed with 4% of PFA for 20 min at
28 RT. After several washes with PBS, cells were stained with HCS Cell Mask™ Blue Stain
29 (Invitrogen, 1:5000) in PBS1X for 20 min and DAPI (1:5000) at RT for 5 min and then
30 mounted. Image stacks (0.5 μm) covering the whole cellular volume were acquired using a
31 confocal-microscope (Zeiss LSM700) with a 63X objective controlled by ZEN software for all
32 co-culture experiments. Images were quantified for (i) the number of acceptor cells which had
33 received the DiD labeled vesicles and (ii) the number of DiD labeled vesicles per acceptor cell.

34 Acceptor cells having DiD vesicles were detected by a script written for detection of cells
35 having two colors (i.e. red and far red for acceptor cells and DiD labeled vesicles respectively).
36 Number of DiD vesicles per acceptor cell were automatically counted by spot detector
37 integrated into the same script. This script produced in ICY software was applied to all co-
38 culture experiments by adjusting the color settings.

39 *Secretion test*

40 To evaluate the possibility of DiD labeled vesicle transfer from donor to acceptor cells mediated
41 by secretion, donor cells were loaded with DiD, washed with 0.01% trypsin, cultivated into
42 fresh medium and incubated for 24 h. Conditioned medium collected from these cells was
43 centrifuged at 1000 rpm for 4 min to remove floating cells and added on acceptor cells. After
44 24 h, cells were fixed with 4% PFA at RT for 20 min. After image acquisition, acceptor cells
45 were counted for the presence of DiD positive vesicles. Secretion test was performed in parallel
46 to all the co-coculture experiments performed in this study by following the same protocol
47 (Supplementary Fig. S4e).

48 *Co-culture preparation for mitochondria transfer experiments*

49 For SW13 cells, donor population were plated on T25 flasks (800.000 cells) and transfected
50 with 3 µg of Mito-GFP for 24 h. Transfected cells were plated on 35 mm dishes and treated
51 with 3 nM tolytoxin or 3 nM methanol for 18 h or left untreated. Meanwhile, acceptor cells
52 were plated on T25 flasks and transfected with 3 µg H2B-mCherry plasmid. For SH-SY5Y
53 cells, stable cell line expressing Mito-DsRed was used as donor population and cells were
54 directly plated on 35 mm dishes and treated with 15 nM tolytoxin or 15 nM methanol for 18h
55 or left untreated. As acceptor cell population, stable cell line expressing H2B-GFP was used.
56 Donor and acceptor cells were mixed in 1:1 ratio (same concentration used in DiD transfer was
57 applied to SW13 cells and 50.000 donors and 50.000 acceptor SH-SY5Y cells were plated on
58 12 mm glass coverslips) and co-cultured for 24 h (Supplementary Figs. S4b, c). After fixation
59 with 4% PFA min at RT for 20, cells were stained for WGA Alexa Fluor©-647 nm (1:300)
60 conjugate and DAPI prior to mounting. After image acquisition, number of acceptor cells,
61 which had received mitochondria and the number of mitochondria received per acceptor cell
62 were quantified. In parallel, secretion of mitochondria was also tested. Briefly, supernatant
63 collected from the conditioned medium of SW13 donor cells (Mito-GFP transfected) and SH-
64 SY5Y donor cells (Mito-DsRed expressing cells) were added onto acceptor cell populations
65 (H2B-mCherry transfected SW13 cells or H2B-GFP expressing SH-SY5Y cells). Acceptor
66 cells were then analysed for the presence of Mito-GFP.
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68 *Co-culture preparation for* α-syn *fibril transfer experiments*

69 1 μM of ATTO-550 tagged α-syn fibrils were diluted in SH-SY5Y cell culture medium and
70 sonicated for 5 min at 80% amplitude with a pulse cycle of 5 seconds on and 2 seconds off in a
71 Vibra-Cell 75041 ultrasonic water bath (Bioblocks Scientic) and immediately transferred on
72 cultured SH-SY5Y and incubated 18 h. Cells were further washed with PBS and 0.01% trypsin
73 (3 times) in order to eliminate non-internalized fibrils, prior to be treated with 15 nM of
74 tolytoxin or methanol for 18 h in parallel to control cells. These cells were used as donor
75 population in co-culture experiments whereas GFP transfected SH-SY5Y cells were used as
76 acceptor population (Supplementary Fig. S4d). After cells were co-cultured for 24 h, they were
77 fixed with 4% PFA 20 min at RT and stained for WGA Alexa Fluor©-647 and DAPI and then
78 mounted. Acceptor cells received α-syn fibrils and the number of α-syn puncta per acceptor cell
79 were then quantified in parallel to secretion test.

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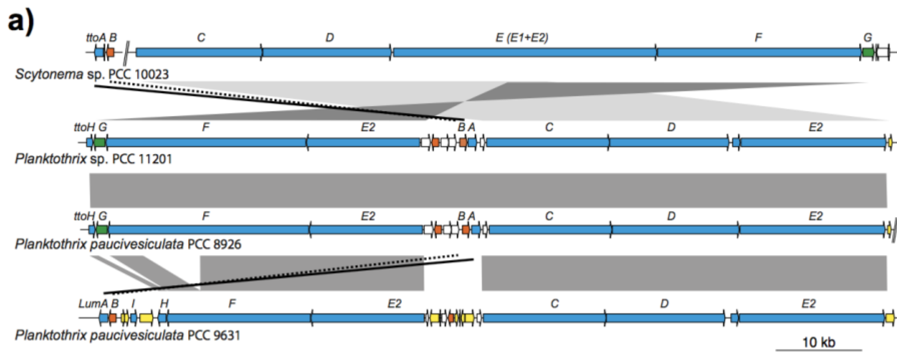
81 **Supplementary Table-1.** Percentage of TNT-connected cells for both cell types. Mean
82 percentage of TNT-connected cells and the standard error of the mean for two sets of TNT
83 counting experiments (three independent experiments for one set of experiment) performed for
84 SW13 and one set of experiment (three independent experiments) performed for SH-SY5Y
85 cells.

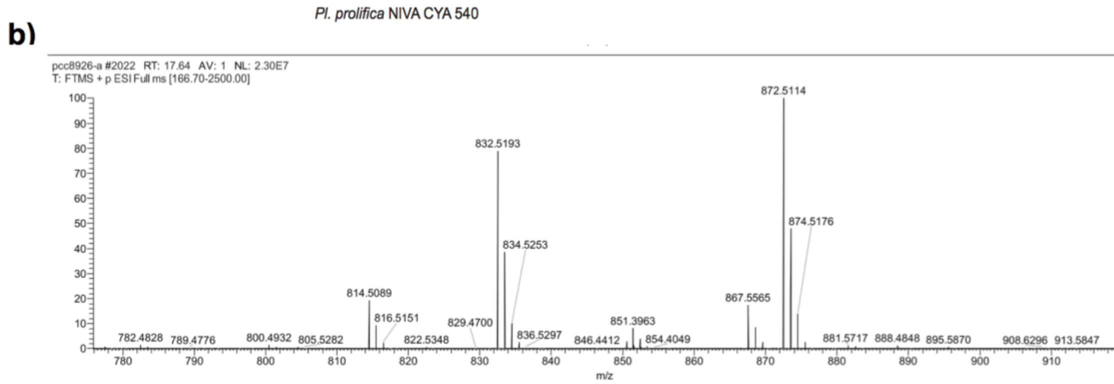
| **SW13** | **TNT-connected cells (%)** |
|---|---|
| Control | 48.37 ± 4.168 |
| 0.5 nM methanol | 46.8 ± 3.998 |
| 0.5 nM tolytoxin | 45.17 ± 4.945 |
| 1.5 nM methanol | 53.9 ± 5.376 |
| 1.5 nM tolytoxin | 40.69 ± 4.251 |
| 3 nM methanol | 50.33 ± 3.604 |
| 3 nM tolytoxin | 29.23 ± 4.371 |
| | |
| Control | 44.24 ± 3.659 |
| 5 nM methanol | 39.8 ± 5.106 |
| 5 nM tolytoxin | 20.19 ± 6.75 |
| 10 nM methanol | 35 ± 9.68 |
| 10 nM tolytoxin | 17.55 ± 5.044 |
| 20 nM methanol | 33.4 ± 7.891 |
| 20 nM tolytoxin | 15.75 ± 5.429 |
| | |
| **SH-SY5Y** | **TNT-connected cells c (%)** |
| Control | 43.16 ± 2.148 |
| 3 nM methanol | 44 ± 5.494 |
| 3 nM tolytoxin | 40.16 ± 4.018 |
| 5 nM methanol | 39.23 ± 3.085 |
| 5 nM tolytoxin | 37.61 ± 3.656 |
| 15 nM methanol | 46.37 ± 2.822 |
| 15 nM tolytoxin | 32.53 ± 2.489 |

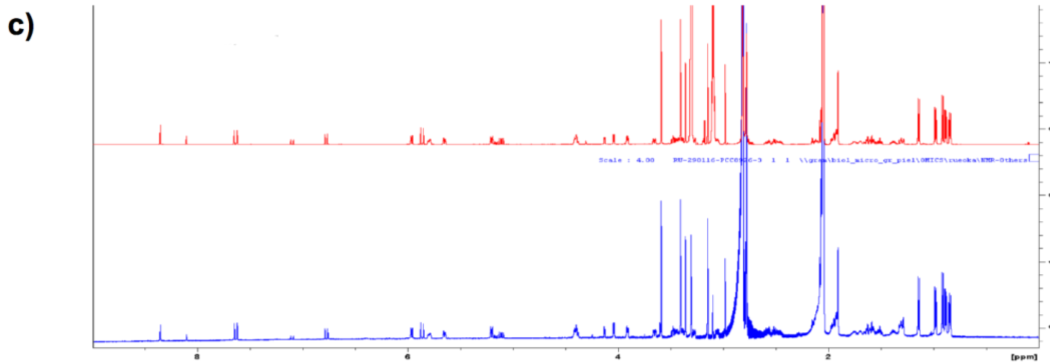
**Supplementary Figure S1.** Comparison of tolytoxin and luminaolide B biosynthesis gene clusters.

**a**) Gene cluster conservation and rearrangement in four strains producing tolytoxin or luminaolide B. Named accordingly to the reference gene cluster of *Scytonema* sp. PCC 10023, the tolytoxin gene clusters include one acyltransferase (*ttoA*), one thioesterase (*ttoH*) and four to five *trans* AT-PKS and (*tto C-F*) indicated in blue, one cytochrome P450 gene (*ttoG*) in green, permease and transporter (brown), transposase (yellow), and other genes with unknown function (white). The grey shadowed areas link conserved genes between the four pathways according to phylogeny of the KS genes, which highlight the rearrangement of the cluster from one strain to another. **b**) Spectrometry and purity check of tolytoxin from *Planktothrix paucivesiculata* PCC 8926, Mass spectrum of the peak at 17.64 min from PCC 8926 ($m/z$ 872.51 $[M+Na]^+$, $m/z$ 832.52 $[M+H-H_2O]^+$, $m/z$ 814.51 $[M+H-2H_2O]^+$). **c)** Purity of the tolytoxin extracted from PCC 8926 used for this study. $^1$H NMR data of tolytoxin (upper) and PCC 8926-Fr.3 (lower) in acetone-$d_6$ at 298 K.

## Supplementary Figure-S1

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126 **Supplementary Figure S2.** Increase in Pan-actin / F-actin ratio in the presence of tolytoxin,
127 TNTs without cargo and having either mitochondria or α-syn in Z-stacks. Effect of tolytoxin
128 on actin cytoskeleton in SKOV-3 cells
129 **a)** F- and Pan-actin labeling for Control, 100 nM Me-control and 100 nM tolytoxin treated
130 SW13 and SHSY-5Y cells. Arrows indicate increased G-actin labelling. Scale bar: 10 µm. **b)**
131 upper graph: Pan Actin/F actin ratio in SW13 cells; Control: 0.28 ± 0.01, Me-Control: 0.58 ±
132 0.02, 3 nM tolytoxin: 1.29 ± 0.17 (p=0.06 for control *versus* 3 nM Me-control, p<0.0001 for
133 control *versus* 3 nM tolytoxin, p<0.0001 for 3 nM Me-control *versus* 3 nM tolytoxin). Lower
134 graph: Pan Actin/F actin ratio in SH-SY5Y cells; Control: 0.55 ± 0.01, Me-Control: 0.6 ± 0.02,
135 15 nM tolytoxin: 0.88 ± 0.04 (p=0.68 for control *versus* 15 nM Me-control, p<0.0001 for
136 control *versus* 15 nM tolytoxin, p<0.0001 for 15 nM Me-control *versus* 15 nM tolytoxin). Data
137 were collected from three independent experiments; 40 cells were analysed per condition. **c)**
138 Sequential image sections of TNTs from upper slices to bottom slice in SW13 and SHSY-5Y
139 cells. Arrows indicate TNTs forming from upper sections and disappearing in bottom sections.
140 Scale bar: 10 µm. **d**) Bottom and upper sections of mitochondria (MitoDsred) and WGA Alexa
141 488 labeled SH-SY5Y cells. White arrow indicates mitochondria inside of a TNT visible only
142 in upper section. Selected area (indicated with white rectangle) is presented in 3D in a different
143 angle, WGA Alexa 488 is pseudo-colored in grey (Left). Bottom and upper sections of Atto
144 550 tagged α-syn fibrils and WGA Alexa 488 labeling in SH-SY5Y cells. White arrow indicates
145 α-syn inside of a TNT visible only in upper section. Selected area (indicated with white
146 rectangle) is presented in 3D in a different angle, WGA Alexa 488 is pseudo-colored in grey in
147 both IF and 3D representation (Right). Scale bars: 10 µm for IF images and 5 µm for 3D
148 presentations). **e)** Orthogonal view of mitochondria transferred into the cytoplasm of an
149 acceptor cell in SH-SY5Y co-culture. Scale bar: 10 µm. **f)** Rhodamine-phalloidin labeling in
150 SKOV-3 cells in Control, 3 nM and 15 nM methanol or tolytoxin treated cells. Arrows indicate
151 intact actin filaments. Scale bar: 20 µm.
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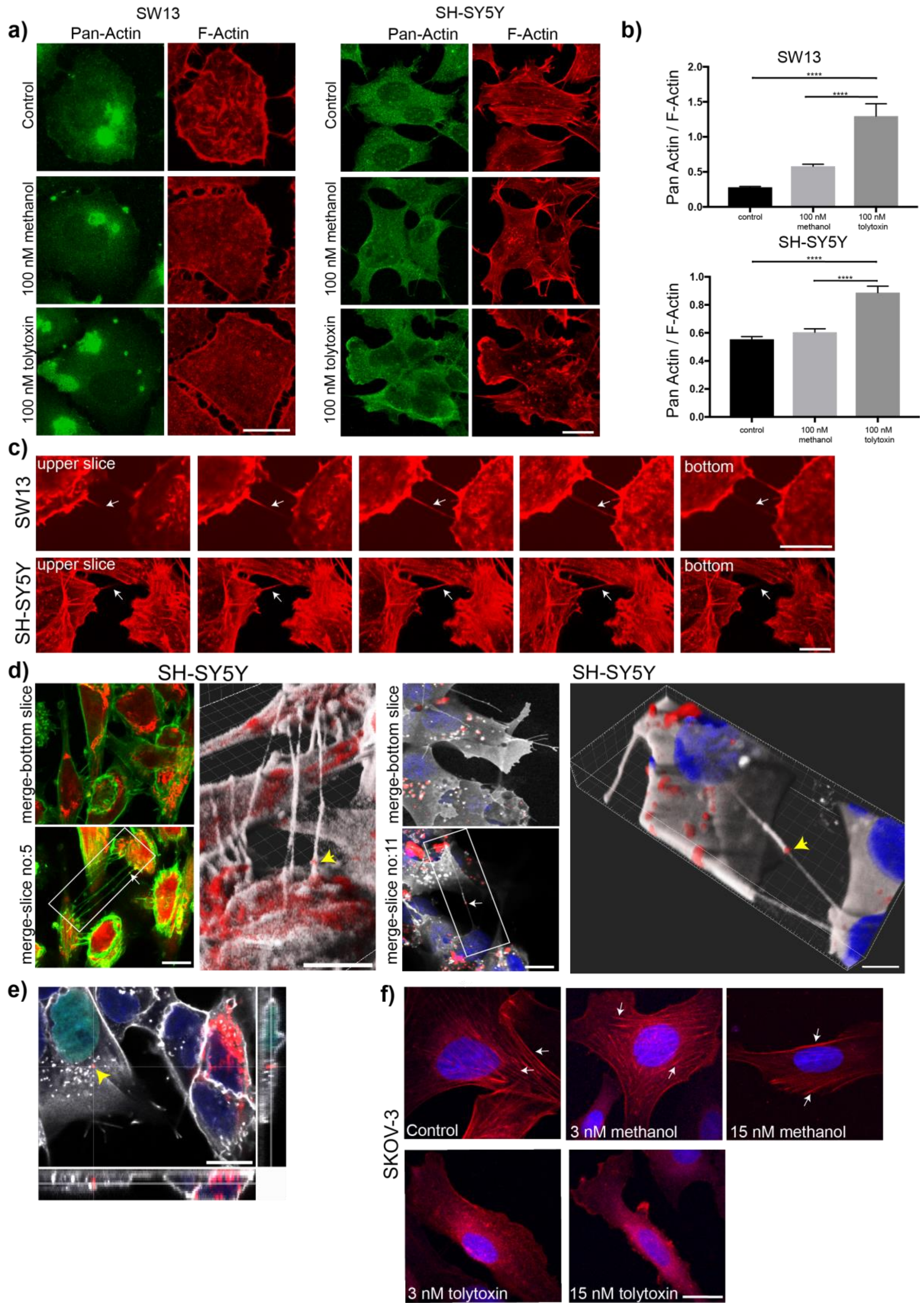
**Supplementary Figure-S2**

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160 **Supplementary Figure S3.** Effect of tolytoxin on filopodia formation.

161 **a)** Attached filopodia detection in SW13 cells. Control, Me-Control and 3 nM tolytoxin treated
162 cells labelled with vinculin, HCS Cell Mask$^{TM}$ Blue Stain and DAPI. **b)** Graph showing the
163 average number of attached filopodia quantification in SW13 cells. Mean number of attached
164 filopodia in Control: 88.32 ± 4.16, Me-control: 78.73 ± 3.17, 3 nM tolytoxin: 86.94 ± 3.08
165 (p=0.141 for control *versus* 3 nM Me-control, p=0.956 for control *versus* 3 nM tolytoxin,
166 p=0.231 for 3 nM Me-control *versus* 3 nM tolytoxin). **c)** Attached filopodia detection in SH-
167 SY5Y cells. Control, Me-Control and 15 nM tolytoxin treated SH-SY5Y cells labelled with
168 vinculin, rhodamine phalloidine and DAPI. **d)** Graph showing the average number of attached
169 filopodia in Control: 43.41 ± 3.89, Me-control: 57,85 ± 6.66, 15 nM tolytoxin: 39.42 ± 3.34
170 (p=0.09 for control *versus* 15 nM Me-control, p=0.832 for control *versus* 15 nM tolytoxin,
171 p=0.06 for 15 nM Me-control *versus* 15 nM tolytoxin). Data were collected from three
172 independent experiments; 40 cells were analysed per condition**.** Scale bar: 10 µm.

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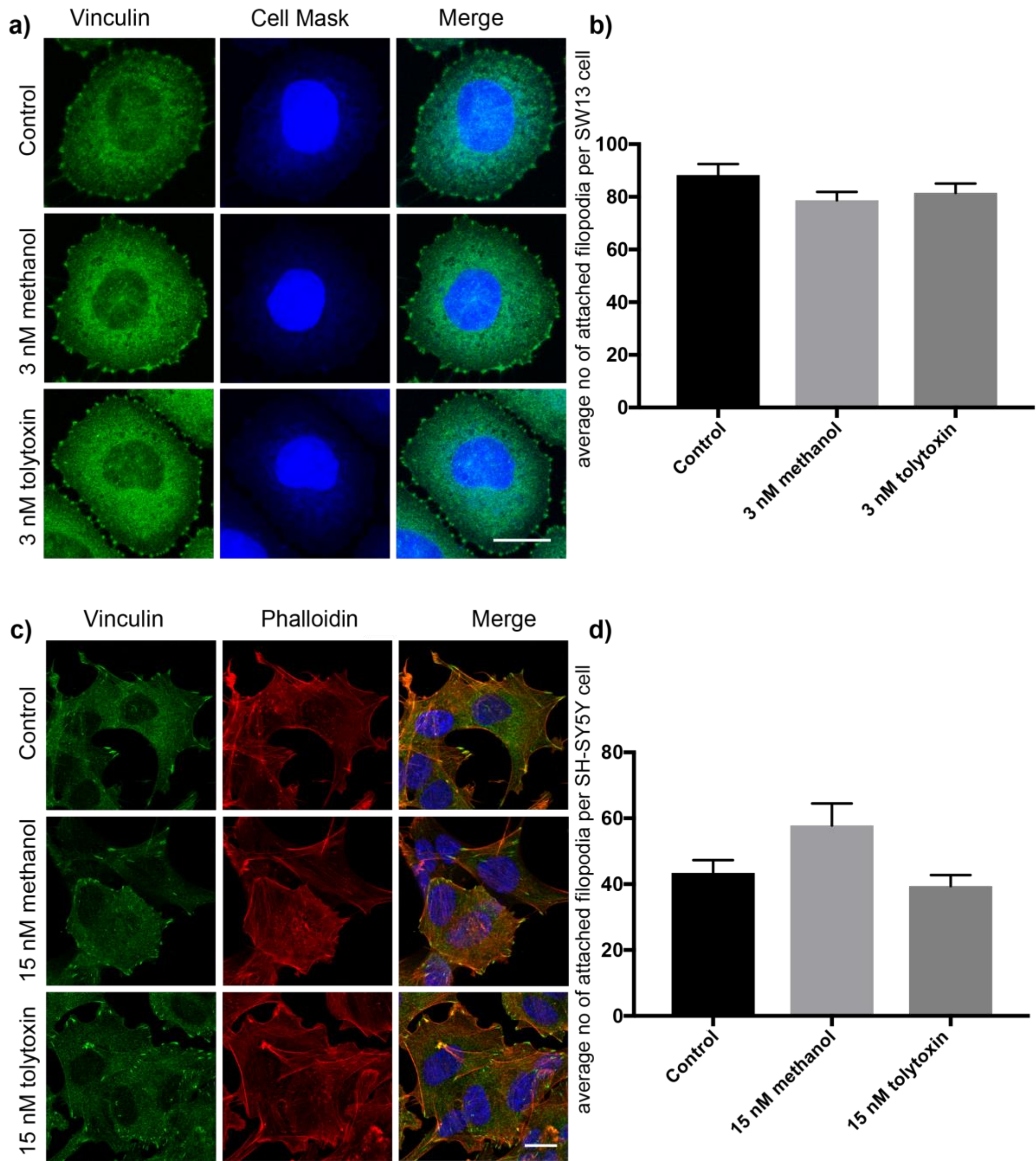
## Supplementary Figure-S3

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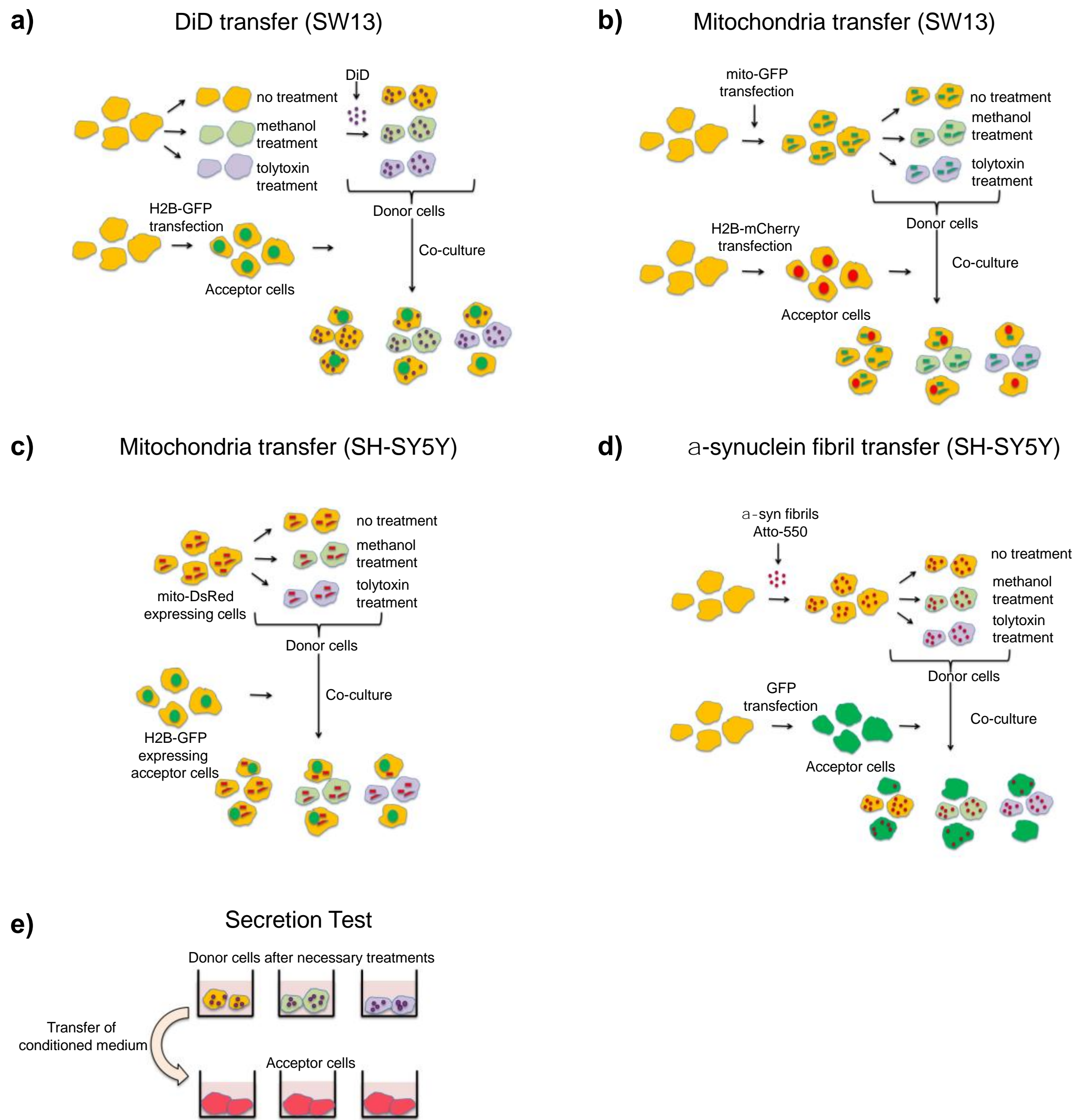
**Supplementary Figure S4.** Schematic of the experimental design of co-culture experiments. **a)** Experimental design for DiD vesicle transfer: Donor SW13 cells were either treated 18 h with 3 nM of methanol or tolytoxin or left untreated. They were labeled with vybrant DiD dye for 20 min and co-cultured with H2B-GFP transfected acceptor cells for 24 h. **b)** Experimental design for mitochondria transfer in SW13 cells: Donor cells were transfected with Mito-GFP plasmid for 24 h and treated either with 3 nM of methanol or tolytoxin or left untreated. They were then co-cultured with H2B-mCherry transfected acceptor cells for 24 h. **c)** Experimental design for mitochondria transfer in SH-SY5Y cells: Mito-Dsred expressing SH-SY5Y donor cells were treated with 15 nM of methanol or tolytoxin or left untreated. They were then co-cultured with H2B-GFP expressing acceptor cells for 24 h. **d)** Experimental design for α-syn fibril transfer: SH-SY5Y donor cells were loaded with Atto-550 tagged α-syn fibrils for 18h and then treated with 15 nM of methanol or tolytoxin or left untreated. They were then co-cultured with GFP transfected acceptor cells for 24 h. **e)** Secretion test: For all the co-culture experiments performed, secretion test was conducted in parallel by collecting the conditioned medium of donor cells described in A to D and transferring on top of the suitable acceptor cell for 24 h.

## Supplementary Figure-S4

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225 **Supplementary Figure S5.** Tolytoxin decreases the DiD labeled vesicle transfer in SW13 cells.
226 **a)** Donor cells loaded with vybrant DiD cell labeling solution in Control, 3 nM Me-control and
227 3 nM tolytoxin co-cultures (left panel), acceptor cells transfected with H2B-mCherry plasmid
228 (middle panel), donor and acceptor cells that were mixed with 1:1 ratio in different co-culture
229 conditions (right panel). Arrows indicate DiD-positive vesicles that had been received by
230 acceptor cells. Scale bar:10 µm. **b)** Left graph showing the percentage of DiD transfer in each
231 co-culture condition. Mean percentage of DiD transfer in control: 63.47% ± 4.80, Me-control:
232 58.81% ± 4.47, 3 nM tolytoxin: 42.4% ± 5.18 (p=0.777 for Control *versus* 3 nM Me-control,
233 p=0.0088 for Control *versus* 3 nM tolytoxin, p=0.0453 for 3 nM Me-control *versus* 3 nM
234 tolytoxin). Right graph showing the average number of DiD vesicles per acceptor cell in each
235 co-culture condition: Control: 12.49% ± 1,58, Me-control: 9.31% ± 0.88, 3 nM tolytoxin:
236 10.74% ± 1,52 (p=0.839 for Control *versus* 3 nM Me-control, p=0.585 for Control *versus* 3 nM
237 tolytoxin, p=0.45 for 3 nM Me-control *versus* 3 nM tolytoxin). Data were collected from three
238 independent experiments; 50 cells were analysed per condition**.**
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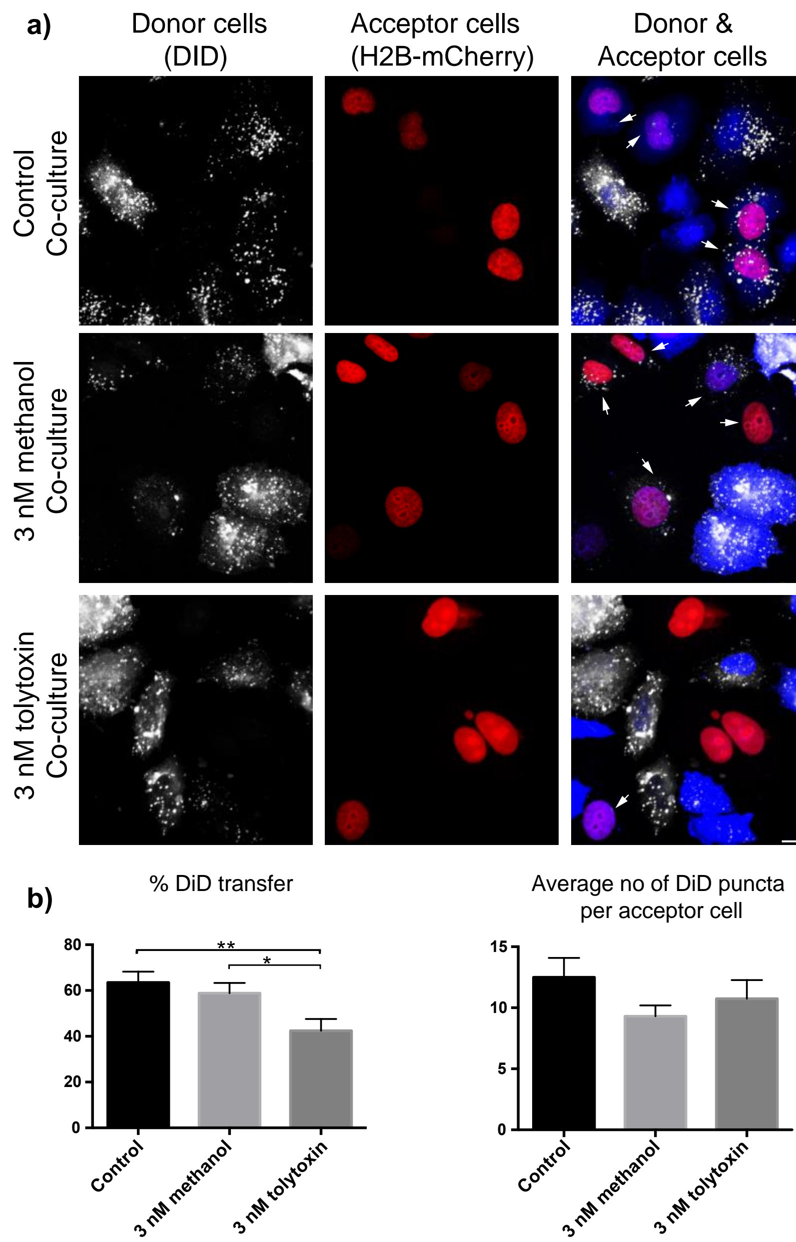
**Supplementary Figure-S5**

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