Tetracyclin-inducible expression of TrkA in human neuroblastoma cell line SY5Y

The ectopic expression of TrkA in neuroblastoma cell lines reduces cell viability and proliferation (1, 2). In cell lines, high ectopic expression often results in significant auto-phosphorylation and auto-activity of TrkA, which puts selective pressure on the transfected cells. This, in turn, causes alterations of the downstream circuits and target genes of TrkA. Additionally, many experiments are not feasible because neuroblastoma cells lose tumorigenicity in mice if stable transfected with TrkA. Hence, to better understand the biological role of TrkA expression in neuroblastoma, an inducible system is required.

In order to set up a vector system for conditional TrkA expression using the tetracyclin repressor, we first optimized the codon sequence of human TrkA cDNA for optimal expression in human cells and obtained a Codon Adaption Index (CAI) of 0.94. A CAI of >0.9 is sufficient to ensure high expression. Furthermore, the GC content of the cDNA was optimized to stabilise the mRNA. Predicted stem-loops, which could negatively influence the binding of the ribosome, were deleted. The optimized human TrkA cDNA given below still codes for the wild type amino acid sequence of TrkA (Fig. 1).

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Optimized     10 ATGCTGCGAGGTGGAAGGAGAGGCCAGCTGGGCTGGCACAGCTGGGCAGCAGGACCTGGAAGCCTGCTGGCATGGCTGATCCTGGCAAGCGCCGGAGCAGCACCCTGCCCCGACGCCTGC
Original      10 ATGCTGCGAGGCGGACGGCGCGGGCAGCTTGGCTGGCACAGCTGGGCTGCGGGGCCGGGCAGCCTGCTGGCTTGGCTGATACTGGCATCTGCGGGCGCCGCACCCTGCCCCGATGCCTGC
Optimized   130 TGCCCCCACGGCAGCAGCGGCCTGCGGTGCACCCGGGACGGCGCCCTGGACAGCCTGCACCACCTGCCCGGCGCCGAGAAACCTGACCGAGCTGTACATCGAAAATCAGCAGCACCTGCAG
Original    130 TGCCCCCACGGCTCCTCGGGACTGCGATGCACCCGGGATGGGGCCCTGGATAGCCTCCACCACCTGCCCGGCGCAGAGAACCTGACTGAGCTCTACATCGAGAACCAGCAGCATCTGCAG
Optimized   250 CACCTGGAGCTGAGAGACCTGAGAGGACTGGGAGAGCTGAGAAACCTGACCATTGTGAAGTCTGGACTGAGATTTGTGGCACCTGACGCCTTCCACTTCACCCCTAGACTGAGCCGGCTG
Original    250 CATCTGGAGCTCCGTGATCTGAGGGGCCTGGGGGAGCTGAGAAACCTCACCATCGTGAAGAGTGGTCTCCGTTTCGTGGCGCCAGATGCCTTCCATTTCACTCCTCGGCTCAGTCGCCTG
Optimized   370 AACCTGAGCTTCAACGCCCTGGAGAGCCTGAGCTGGAAGACCGTGCAGGGACTGAGCCTGCAGGAGCTGGTGCTGTCTGGAAACCCACTGCACTGTTCCTGTGCACTGAGATGGCTGCAG
Original    370 AATCTCTCCTTCAACGCTCTGGAGTCTCTCTCCTGGAAAACTGTGCAGGGCCTCTCCTTACAGGAACTGGTCCTGTCGGGGAACCCTCTGCACTGTTCTTGTGCCCTGCGCTGGCTACAG
Optimized   490 CGGTGGGAGGAGGAGGGACTGGGCGGCGTGCCAGAGCAGAAGCTGCAGTGCCACGGCCAGGGCCCCCTGGCCCACATGCCCAATGCCAGCTGTGGTGTGCCCACGCTGAAGGTCCAGGTG
Original    490 CGCTGGGAGGAGGAGGGACTGGGCGGAGTGCCTGAACAGAAGCTGCAGTGTCATGGGCAAGGGCCCCTGGCCCACATGCCCAATGCCAGCTGTGGTGTGCCCACGCTGAAGGTCCAGGTG
Optimized   610 CCAAATGCATCTGTGGACGTGGGAGATGACGTGCTGCTGAGATGCCAGGTGGAGGGAAGAGGACTGGAGCAGGCAGGCTGGATCCTGACCGAGCTGGAGCAGCCGCCACAGTGATGAAA
Original    610 CCCAATGCCTCGGTGGATGTGGGGGACGACGTGCTGCTGCGGTGCCAGGTGGAGGGGCGGGGCCTGGAGCAGGCCGGCTGGATCCTCACAGAGCTGGAGCAGTCAGCCACGGTGATGAAA
Optimized   730 AGCGGCGGCCTGCCAAGCCTGGGACTGACCCTGGCAAACGTGACCAGCGACCTGAACCGGAAGAACGTGACCTGCTGGGCAGAGAATGACGTGGGAAGAGCAGAGGTGTCTGTGCAGGTG
Original    730 TCTGGGGGTCTGCCATCCCTGGGGCTGACCCTGGCCAATGTCACCAGTGACCTCAACAGGAAGAACGTGACGTGCTGGGCAGAGAACGATGTGGGCCGGGCAGAGGTCTCTGTTCAGGTC
Optimized   850 AACGTGAGCTTCCCAGCATCTGTGCAGCTGCACACAGCAGTGGAGATGCACCACTGGTGCATTCCTTTCTCTGTGGACGGACAGCCTGCACCAAGCCTGAGATGGCTGTTCAATGGAAGC
Original    850 AACGTCTCCTTCCCGGCCAGTGTGCAGCTGCACACGGCGGTGGAGATGCACCACTGGTGCATCCCCTTCTCTGTGGATGGGCAGCCGGCACCGTCTCTGCGCTGGCTCTTCAATGGCTCC
Optimized   970 GTGCTGAATGAGACCAGCTTCATCTTCACCGAGTTCCTGGAGCCTGCAGCAAATGAAACCGTGCGGCACGGATGCCTGAGACTGAACCAGCCAACCCACGTGAACAACGGCAACTACACA
Original    970 GTGCTCAATGAGACCAGCTTCATCTTCACTGAGTTCCTGGAGCCGGCAGCCAATGAGACCGTGCGGCACGGGTGTCTGCGCCTCAACCAGCCCACCCACGTCAACAACGGCAACTACACG
Optimized  1090 CTGCTGGCAGCAAACCCCTTCGGCCAGGCAAGCGCCAGCATCATGGCAGCATTCATGGACAACCCCTTCGAGTTCAACCCCGAGGACCCCATCCCGTGAGCTTCAGCCCCGTGGACACC
Original   1090 CTGCTGGCTGCCAACCCCTTCGGCCAGGCCTCCGCCTCCATCATGGCTGCCTTCATGGACAACCCTTTCGAGTTCAACCCCGAGGACCCCATCCCTGTCTCCTTCTCGCCGGTGGACACT
Optimized  1210 AACAGCACCAGCGGAGACCCTGTGGAGAAGAAGGATGAAACACCCTTCGGCGTGAGCGTGGCAGTGGGACTGGCCGTGTTCGCATGCCTGTTCCTGAGCACCCTGCTGCTGGTGCTGAAC
Original   1210 AACAGCACATCTGGAGACCCGGTGGAGAAGAAGGACGAAACACCTTTTGGGGTCTCGGTGGCTGTGGGCCTGGCCGTCTTTGCCTGCCTCTTCCTTTCTACGCTGCTCCTTGTGCTCAAC
Optimized  1330 AAGTGCGGAAGGAGAAACAAATTTGGAATCAATAGACCTGCCGTGCTGGCACCTGAGGATGGACTGGCAATGAGCCTGCACTTCATGACCCTGGGCGGCAGCAGCCTGAGCCCAACAGAG
Original   1330 AAATGTGGACGGAGAAACAAGTTTGGGATCAACCGCCCGGCTGTGCTGGCTCCAGAGGATGGGCTGGCCATGTCCCTGCATTTCATGACATTGGGTGGCAGCTCCCTGTCCCCCACCGAG
Optimized  1450 GGAAAGGGATCTGGACTGCAGGGACACATCATTGAGAACCCACAGTACTTCAGCGACGCCTGCGTGCACCACATCAAGCGGCGGGACATCGTGCTGAAGTGGGAGCTGGGCGAGGGCGCC
Original   1450 GGCAAAGGCTCTGGGCTCCAAGGCCACATCATCGAGAACCCACAATACTTCAGTGATGCCTGTGTTCACCACATCAAGCGCCGGGACATCGTGCTCAAGTGGGAGCTGGGGGAGGGCGCC
Optimized  1570 TTCGGCAAGGTGTTCCTGGCCGAGTGCCACAACCTGCTGCCCGAGCAGGACAAGATGCTGGTGGCAGTGAAGGCCCTGAAAGAGGCATCTGAGAGCGCCAGACAGGACTTCCAGAGAGAG
Original   1570 TTTGGGAAGGTCTTCCTTGCTGAGTGCCACAACCTCCTGCCTGAGCAGGACAAGATGCTGGTGGCTGTCAAGGCACTGAAGGAGGCGTCCGAGAGTGCTCGGCAGGACTTCCAGCGTGAG
Optimized  1690 GCAGAGCTGCTGACCATGCTGCAGCACCAGCACATCGTGCGGTTCTTCGGCGTGTGCACCGAGGGAAGACCTCTGCTGATGGTGTTCGAGTACATGCGGCACGGCGACCTGAACCGGTTC
Original   1690 GCTGAGCTGCTCACCATGCTGCAGCACCAGCACATCGTGCGCTTCTTCGGCGTCTGCACCGAGGGCCGCCCCCTGCTCATGGTCTTTGAGTATATGCGGCACGGGGACCTCAACCGCTTC
Optimized  1810 CTGCGGAGCCACGGCCCCGACGCCAAGCTGCTGGCCGGCGGCGAGGACGTGGCACCTGGACCACTGGGACTGGGACAGCTGCTGGCAGTGGCCAGCCAGGTGGCCGCCGGCATGGTGTAC
Original   1810 CTCCGATCCCATGGACCTGATGCCAAGCTGCTGGCTGGTGGGGAGGATGTGGCTCCAGGCCCCCTGGGTCTGGGGCAGCTGCTGGCCGTGGCTAGCCAGGTCGCTGCGGGGATGGTGTAC
Optimized  1930 CTGGCAGGACTGCACTTTGTGCACAGAGACCTGGCAACCAGAAACTGCCTGGTGGGACAGGGACTGGTGGTGAAGATCGGCGACTTCGGCATGAGCCGGGACATCTACAGCACCGACTAC
Original   1930 CTGGCGGGTCTGCATTTTGTGCACCGGGACCTGGCCACACGCAACTGTCTAGTGGGCCAGGGACTGGTGGTCAAGATTGGTGATTTTGGCATGAGCAGGGATATCTACAGCACCGACTAT
Optimized  2050 TACCGGGTGGGCGGCCGGACCATGCTGCCCATCCGGTGGATGCCTCCAGAGAGCATCCTGTACCGGAAGTTCACCACCGAGAGCGACGTGTGGAGCTTCGGCGTGGTGCTGTGGGAGATC
Original   2050 TACCGTGTGGGAGGCCGCACCATGCTGCCCATTCGCTGGATGCCGCCCGAGAGCATCCTGTACCGTAAGTTCACCACCGAGAGCGACGTGTGGAGCTTCGGCGTGGTGCTCTGGGAGATC
Optimized  2170 TTCACCTACGGCAAGCAGCCCTGGTACCAGCTGAGCAACACCGAGGCCATCGACTGCATCACCCAGGGCCGGGAGCTGGAGCGGCCTAGAGCTTGCCCTCCAGAGGTGTATGCAATCATG
Original   2170 TTCACCTACGGCAAGCAGCCCTGGTACCAGCTCTCCAACACGGAGGCAATCGACTGCATCACGCAGGGACGTGAGTTGGAGCGGCCACGTGCCTGCCCACCAGAGGTCTACGCCATCATG
Optimized  2290 AGAGGATGCTGGCAGAGAGCCACAGCAGAGACACAGCATCAAGGACGTGCACGCCCGGCTGCAGGCCCTGGCCCAGGCTCCTCCAGTGTACCTGGACGTGCTGGGCTGA
Original   2290 CGGGGCTGCTGGCAGCGGGAGCCCCAGCAACGCCACAGCATCAAGGATGTGCACGCCCGGCTGCAAGCCCTGGCCCAGGCACCTCCTGTCTACCTGGATGTCCTGGGCTAG
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Fig. 1: Sequence of wild type and codon optimized TrkA. Altered codons in the optimized sequence are highlighted in red.

We next cloned this optimized human TrkA cDNA (“TrkAcodop”) into pT-REx-DEST30 (Invitrogen, Carlsbad, CA), a vector for Tet-conditional expression. We transfected the human neuroblastoma cell line SY5Y with pcDNA6/TR (Invitrogen), which harbours the gene coding for the tetracyclin repressor, and raised single cell clones (SY5Y-TR) by limited dilution and antibiotic selection (blasticidine). We then transfected SY5Y-TR with pT-REx-DEST30-TrkAcodop. By continued selection with antibiotics (neomycin, blasticidine was also continued to maintain TR) and performing a limited dilution we generated six clonally derived cell lines (designated SY5Y-TR-TrkAcodop 1-6). In the absence of Tetracyclin, SY5Y-TR-TrkAcodop does not

express TrkA, while upon addition of Tetracylin, TrkA is induced and expressed as long as Tetracylin is present. All cell lines were shown to be free of mycoplasma and authenticated at DSMZ (German Collection of Microorganisms and Cell Cultures, Germany, Braunschweig). This confirmed that the cell lines SY5Y, SY5Y-TR (expressing the tetracyclin repressor gene) and SY5Y-TR-TrkA clone 1 (expressing TrkA after tetracyclin treatment) match with the human neuroblastoma cell line SY5Y.

Expression of the human codon optimized TrkA was confirmed by RT-qPCR (Primer: sen *TCGAAAATCAGCAGCACC*; rev *GCCACAAATCTCAGTCCA*). Following incubation with tetracyclin (1µg/ml) or doxycyclin (1µg/ml) for 48h TrkA expression was detected only in Tet-treated SY5Y-TrkA clones 1-6, but not in parental SY5Y, SY5Y-TR (harbouring only the tetracyclin repressor) or cells without treatment (Fig. 2). Induced expression of TrkA could also been shown on protein level using Western Blotting (α-TrkA Santa Cruz, Santa Cruz, CA, sc-11) for clone 1,4 and 6 (Fig. 3). Most interestingly, activation of TrkA occurred only in the presence of the TrkA-ligand, NGF (100 ng/ml). High expression of TrkA did not result in autophosphorylation when cells were treated with tetracyclin (1µg/ml) only (Fig. 4).

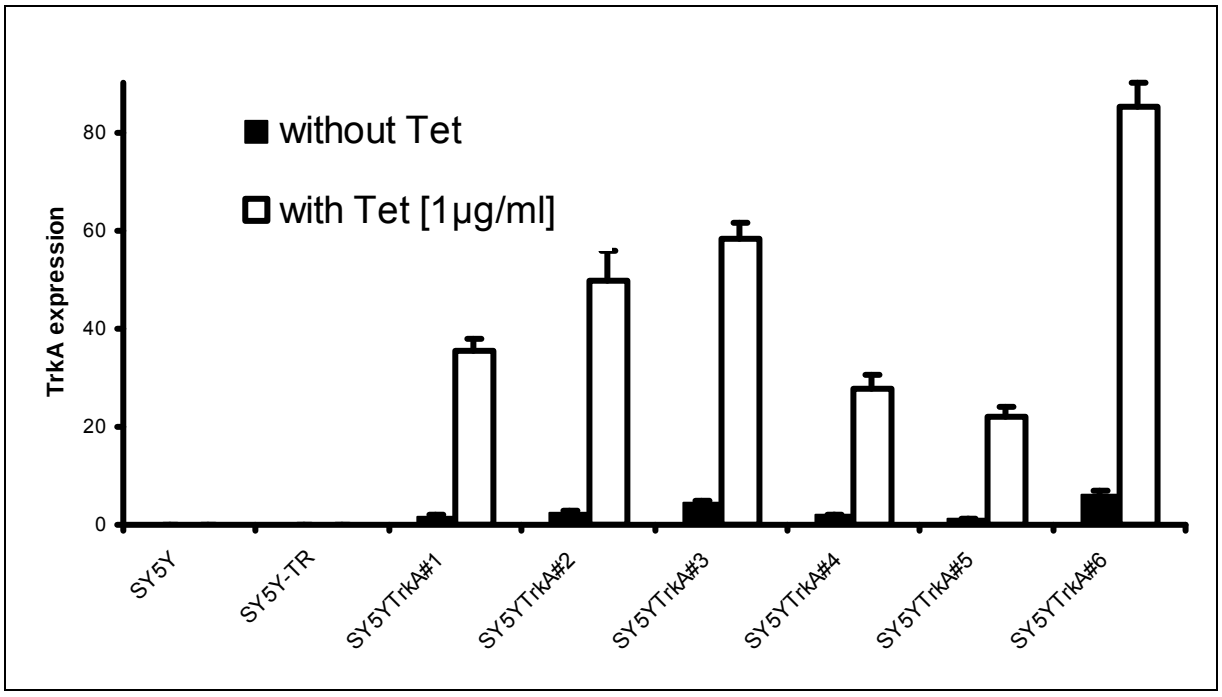

Fig. 2: RT-qPCR analysis relvealed high and stable expression of TrkA after 72h treatment with tetracyclin. The SY5Y-TrkA clones 1-6, which were not treated with tetracyclin show only little higher basal expression of TrkA compared to SY5Y and SY5Y-TR.

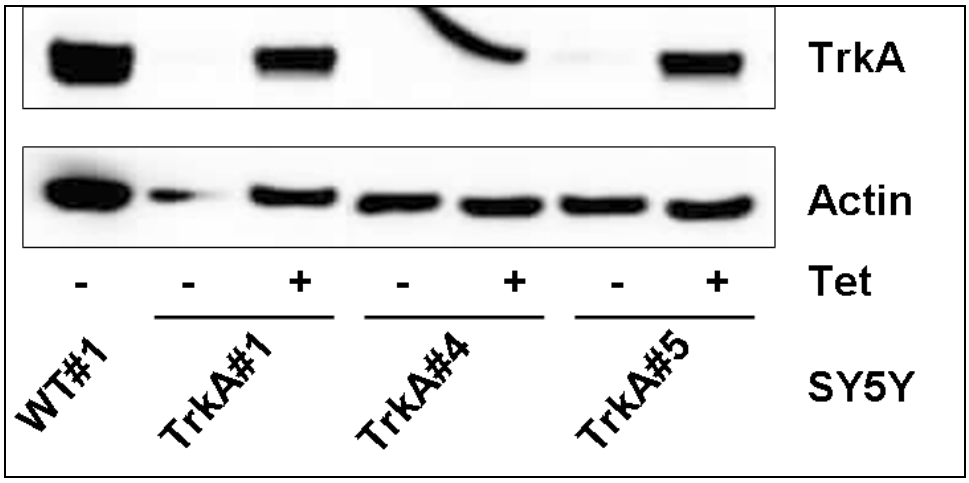

Fig. 3: Induced protein expression of TrkA following tetracyclin treatment. TrkA is only expressed in cells, which were treated with tetracyclin. SY5Y-TrkA-WT#1, expressing TrkA constitutively was used as a control.

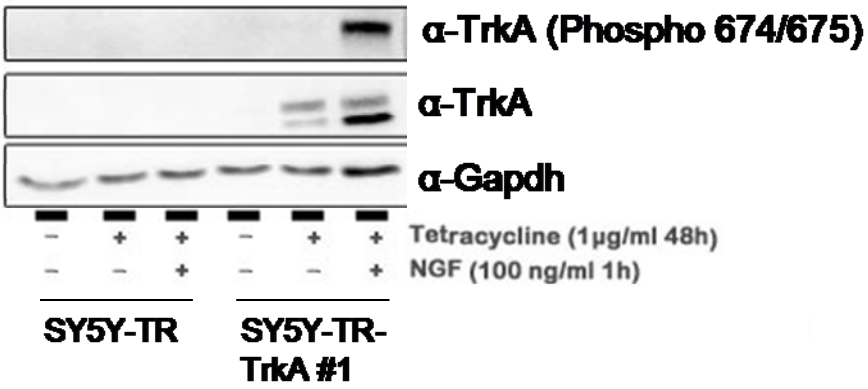

Fig 4: Expression of TrkA can be achieved by addition of Tet, but TrkA-activation requires additional stimulation by NGF, Neither TrkA expression nor activation can be found in the control cell line SY5Y-TR.

Induced expression of TrkA following tetracyclin and NGF (100ng/ml) for the times indicated, SY5Y-TrkA clones show clearly neurite outgrowth and reduced cell number both hinting at induction of differentiation (Fig. 5).

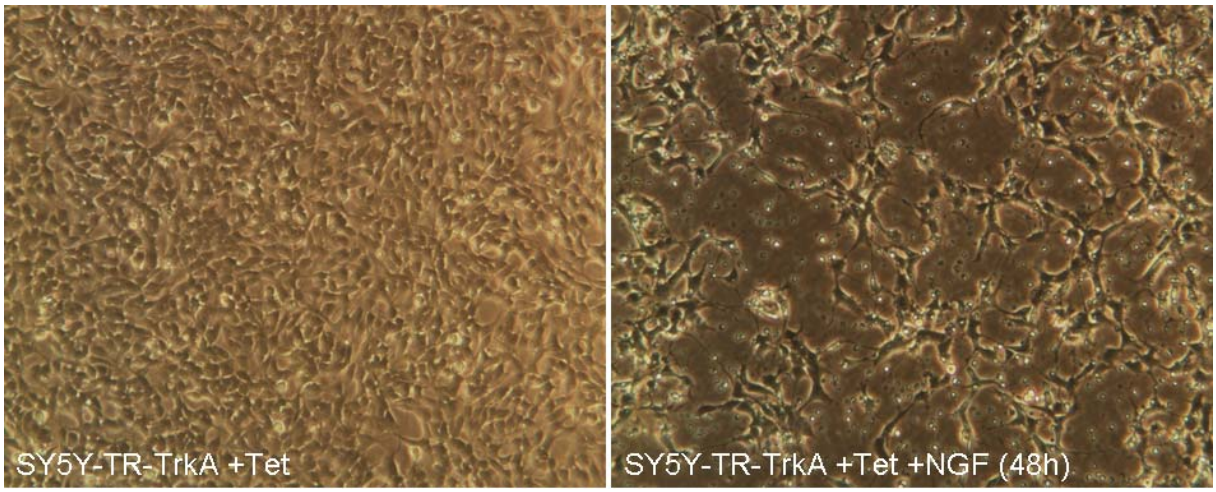

Fig. 5: Tetracyclin induced expression of TrkA and subsequent treatment with its ligand NGF induces morphological signs of differentiation in SY5Y-TR-TrkA clones (shown for one clone, additional data for the remaining cell clones are available upon request). Pls note neurite outgrowth and reduced cell number in the treated cells compared to the untreated control.

CONTACT

Sven Lindner
University Children's Hospital Essen
Hufelandstr.55
45122 Essen
Tel.: ++492017232504
Email: johannes.schulte@uni-due.de

Prof. Dr. Johannes H. Schulte
University Children's Hospital Essen
Hufelandstr.55
45122 Essen
Tel.: ++4920172385185
Email: johannes.schulte@uni-due.de

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