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RESEARCH HIGHLIGHT

The enemy from within: mislocalization of a compromised receptor as a mechanism for TrkAIII oncogenic activity

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There is growing evidence that the mislocalisation of receptor tyrosine kinase oncogenes underpins downstream oncogenic signalling. Here, we highlight our recent work characterising the mechanism that underpins mislocalisation and subsequent oncogenic activity of the alternative TrkAIII splice variant of the tropomyosin related kinase A (TrkA) receptor, in human neuroblastoma cells. In primary neuroblastomas, expression of fully spliced TrkA associates with low-stage disease and better prognosis, whereas TrkAIII expression associates with advanced-stage disease and worse prognosis. In neuroblastoma models TrkA and TrkAIII exhibit opposite tumour suppressing and oncogenic activity, respectively. In an attempt to further understand the basis of this diametrically opposite behaviour, intracellular trafficking and activation TrkA and TrkAIII receptors was compared in SH-SY5Y neuroblastoma cells. We found that TrkAIII oncogenic activity originates from miss-localisation and spontaneous activation within the alternative membrane substrate context of the endoplasmic reticulum-Golgi intermediate (ERGIC)-COP-I vesicle compartment. This results from altered trafficking caused by interphase restricted spontaneous receptor activation, which impedes TrkAIII transport from the ERGIC to the Golgi network, associated with retrograde transport of activated TrkAIII from the ERGIC back to the endoplasmic reticulum (ER). Therefore, spontaneous TrkAIII activation within ERGIC/COPI membranes, facilitated by omission of the extracellular D4 spontaneous activation-prevention domain, sets-up self-perpetuating TrkAIII recycling between the ER and ERGIC. This mechanism ensures continuous accumulation of this compromised receptors above the spontaneous activation threshold of the ERGIC/COPI compartment, resulting in oncogenic signalling through IP3K from this altered substrate context. Furthermore, chronic ER stress caused by TrkAIII recycling back to the ER induces a protective ER-stress response, and also the recruitment of active TrkAIII to the centrosome, altering centrosome behaviour. These different tumour-promoting mechanisms all result from mislocalization and spontaneous activation of TrkAIII within the alternative substrate context of the ERGIC/COPI compartment and can be prevented by TrkA tyrosine kinase inhibitors.

Keywords: neuroblastoma; alternative TrkAIII splice variant; spontaneous activation; retrograde transport; ERGIC; oncogenic activity; receptor mislocalization

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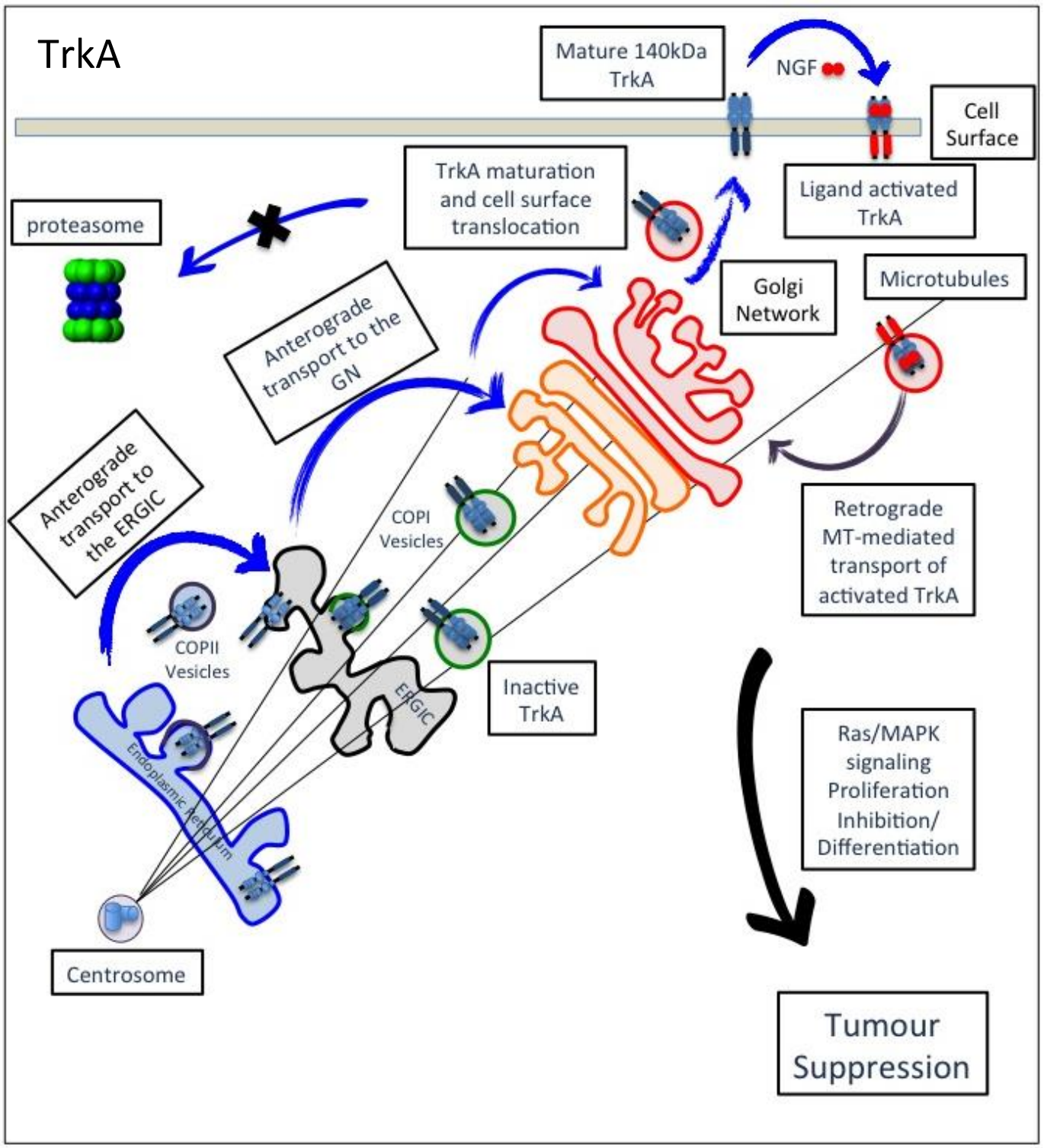
In the pediatric tumor neuroblastoma (NB), expression of the fully spliced NGF receptor tropomyosin-related kinase A (TrkA) correlates with low-stage disease, better prognosis and potential for spontaneous remission. However, expression of the alternative TrkAIII splice variant, discovered in our laboratory in advanced stage metastatic primary NB, has now been confirmed to correlate with advanced-stage metastatic disease, worse prognosis and post-therapeutic disease relapse [1-4]. In NB models, TrkA exhibits tumor-suppressing activity, whereas TrkAIII exhibits oncogenic activity. We characterized TrkAIII as a mislocalized oncoprotein, providing a novel alternative to the classical concept of cell surface receptor tyrosine kinase oncogenic signaling and fuelling the growing concept that receptor tyrosine kinase oncogene mislocalization underpins downstream oncogenic signaling [1, 5].

TrkAIII is characterized by exons 6, 7 and 9 skipping and is expressed as a variant receptor, devoid of the extracellular receptor D4 Ig-like spontaneous activation-prevention domain and several N-glycosylation sites implicated in cell surface receptor expression. It is developmentally regulated [6] and stress-regulated in normal neural crest progenitors, normal neural stem cells and NB tumor cells but not differentiated counterparts [1]. In contrast to fully spliced TrkA receptors, TrkAIII is not expressed at the cell surface but is miss-localized to intracellular membranes, within which it exhibits spontaneous ligand-independent activation, inducing oncogenic signaling through IP3K but not Ras/MAPK from this altered substrate context [1, 7]. This differs to tumor suppressing signaling through Ras/MAPK from ligand-activated cell surface TrkA receptors, which inhibits NB cell proliferation and induces neural differentiation [1, 2]. We consider, therefore, that alternative TrkAIII splicing represents a regulated tumor promoting switch that is characterized by a change from tumor suppressing TrkA signaling to tumor promoting TrkAIII signaling within the stressful NB microenvironment [1, 2].

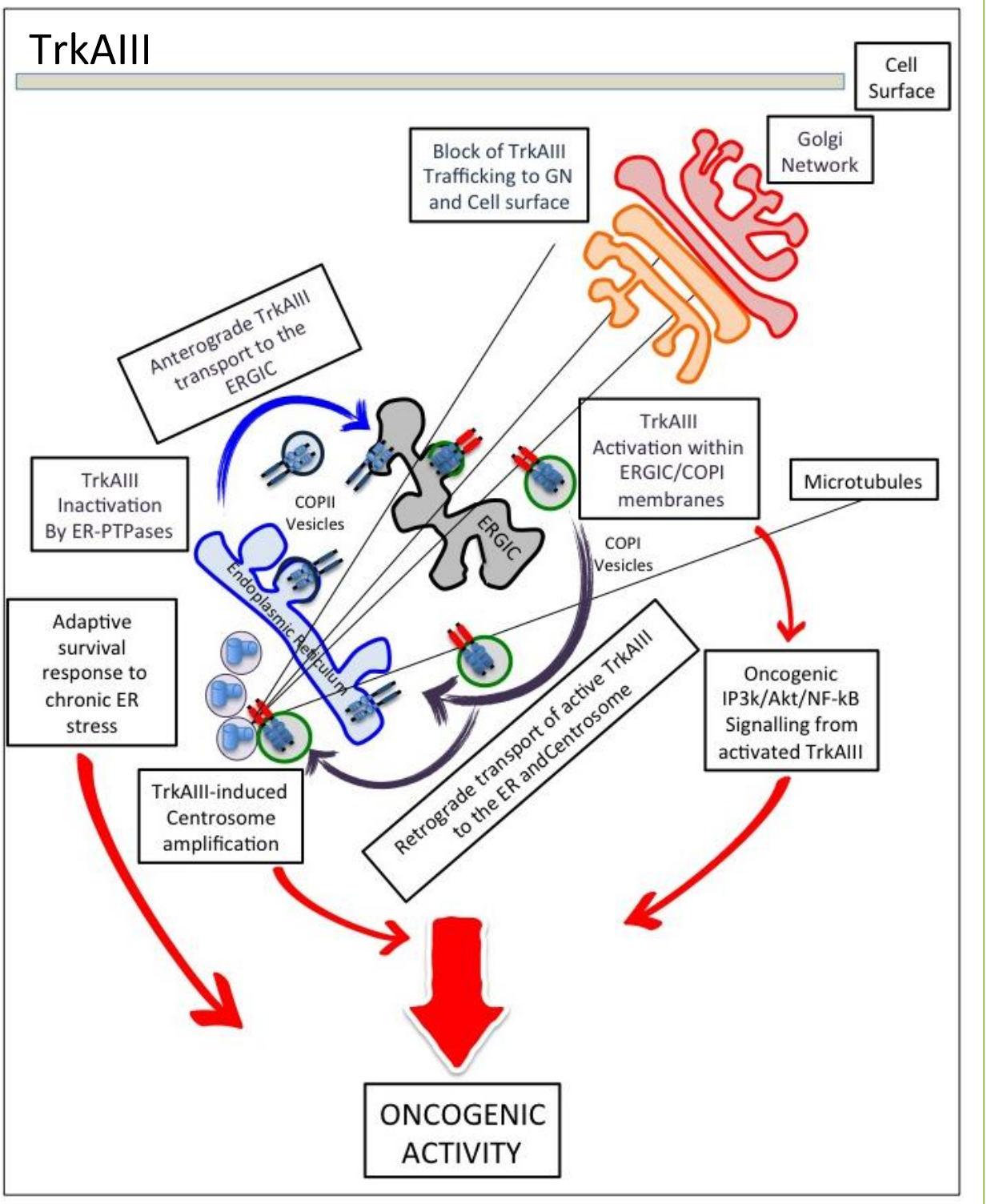
In our recent Oncotarget article [7], we further examined the mechanistic basis for TrkAIII miss-localization and subsequent diametrically opposite behavior to TrkA. By tracking metabolically-labelled receptors, we identified differences in intracellular TrkA and TrkAIII trafficking. Consistent with previous reports, de novo synthesized TrkA was found to exit the endoplasmic reticulum (ER) in immature N-glycosylated 110kDa-form, move rapidly through the endoplasmic reticulum-Golgi intermediate (ERGIC)-COP-I vesicle compartment in an anterograde direction to the Golgi network (GN), where 110kDa TrkA is matured to the 140kDa receptor prior to being transported to the cell surface. This process does not associate with spontaneous ligand-independent activation of either intracellular or cell surface TrkA, despite significant accumulation within both GN and cell surface but not ER, ERGIC or COP vesicle membranes [1, 7]. We attribute the maintenance of an inactive TrkA status to the presence of receptor extracellular D4 and D5 spontaneous activation-prevention domains [8], to PTPase activity within ER, ERGIC, COP-vesicle and cell surface membranes; and to PTPase-independent mechanisms within the GN [7]. We also consider that the potential for spontaneous intracellular TrkA activation is further reduced by the relative velocity of intracellular trafficking, resulting in low levels of accumulation below the threshold for spontaneous activation in ER, ERGIC and COP vesicle compartments [7]. TrkAIII was also found to exit the ER in immature 100kDa N-glycosylated form and move to the ERGIC compartment but in contrast to TrkA, failed to adequately reach the GN, preventing further maturation and imprisoning TrkAIII within pre-GN membrane compartments. This effect was dependent upon spontaneous TrkAIII activation within the ERGIC/COP-I vesicle compartment and was reversed by CEP-701, Go6976 and GW441756 TrkA tyrosine kinase inhibitors. The inhibition of TrkAIII activity also resulted in anterograde transport of inactive TrkAIII from the ERGIC to the GN. This, in turn, promoted 120kDa TrkAIII maturation but did not result in cell surface TrkAIII expression. Indeed, 120kDa TrkAIII matured within the GN under TrkA inhibitory conditions was more sensitive to degradation at the proteasome than either 100kDa immature TrkAIII, 110kDa immature TrkA or 140kDa mature TrkA receptors and was only detected at the cell surface under proteasome inhibitory conditions [7]. This difference in susceptibility to proteasome degradation implicates the TrkA N-glycosylation sites absent in TrkAIII in protecting mature TrkA receptors from proteasome degradation, consistent with a previous report that these sites are required for cell surface TrkA receptor expression [9].

In our Oncotarget article [7], we also found that spontaneous intracellular TrkAIII activation was prevented by the inhibitor of COPI vesicle formation Brefeldin A, and co-localized with ERGIC and COP-I markers in membrane purification and confocal microscopy studies, identifying the ERGIC/COPI membrane compartment as permissive for spontaneous TrkAIII activation. This adds to reports of TrkT3 oncogene activation within COPII membranes and aberrant activation of immature TrkA receptors within ERGIC membranes in SV40 immortalized human kidney epithelial cells [10, 11]. This suggests that ERGIC/COP vesicle membranes, in contrast to ER and GN membranes, have a lower threshold for spontaneous activation of TrkA receptors, dependent upon accumulation. In the case of both TrkAIII and TrkT3 oncoproteins, this potential is augmented by the omission of extracellular spontaneous activation-prevention domains [8].

a


TrkA
Mature 140kDa TrkA
NGF
Cell Surface
Ligand activated TrkA
TrkA maturation and cell surface translocation
proteasome
Golgi Network
Microtubules
Anterograde transport to the GN
Anterograde transport to the ERGIC
COPI Vesicles
COPII Vesicles
ERGIC
Endoplasmic Reticulum
Inactive TrkA
Retrograde MT-mediated transport of activated TrkA
Ras/MAPK signaling Proliferation Inhibition/ Differentiation
Centrosome
Tumour Suppression

b


TrkAIII
Cell Surface
Golgi Network
Block of TrkAIII Trafficking to GN and Cell surface
Anterograde TrkAIII transport to the ERGIC
TrkAIII Activation within ERGIC/COPI membranes
Microtubules
TrkAIII Inactivation By ER-PTPases
COPII Vesicles
ERGIC
COPI Vesicles
Endoplasmic Reticulum
Adaptive survival response to chronic ER stress
Oncogenic IP3k/Akt/NF-kB Signalling from activated TrkAIII
Retrograde transport of active TrkAIII to the ER andCentrosome
TrkAIII-induced Centrosome amplification
ONCOGENIC ACTIVITY

C

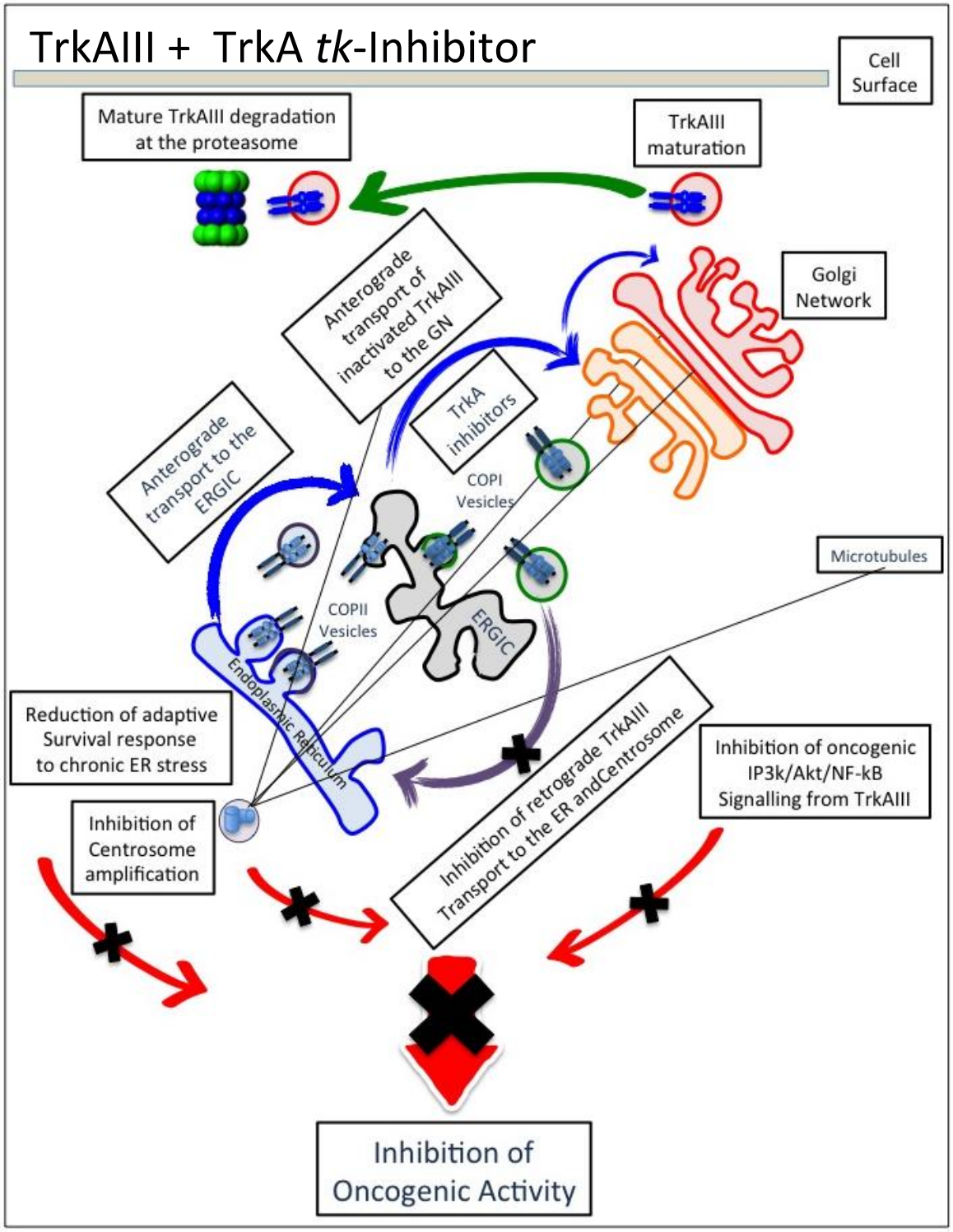

TrkAIII + TrkA tk-Inhibitor
Cell Surface
Mature TrkAIII degradation at the proteasome
TrkAIII maturation
Anterograde transport of inactivated TrkAIII to the GN
Golgi Network
TrkA inhibitors
Anterograde transport to the ERGIC
COPI Vesicles
Microtubules
COPII Vesicles
ERGIC
Endoplasmic Reticulum
Reduction of adaptive Survival response to chronic ER stress
Inhibition of retrograde TrkAIII Transport to the ER andCentrosome
Inhibition of oncogenic IP3k/Akt/NF-kB Signalling from TrkAIII
Inhibition of Centrosome amplification
Inhibition of Oncogenic Activity

Figure 1.Summary of the differences in intracellular TrkA and TrkAIII trafficking, spontaneous activation, post receptor signaling and tumor suppressing verses oncogenic behavior. a) Representation of the intracellular trafficking of fully spliced TrkA receptors, showing anterograde transport of inactive immature *de novo* synthesized TrkA from the ER to ERGIC then GN, where it undergoes N-glycan maturation prior to being transported to the cell surface. The effect of ligand (NGF) activation of cell surface TrkA on motor protein-mediate MT minus-end directed retrograde transport of internalized activated receptor and tumor suppressing post-receptor signaling through Ras/MAPK, is also shown. b) Representation of the intracellular trafficking of the alternative TrkAIII splice variant, showing anterograde transport of inactive immature *de novo* synthesized TrkAIII from the ER to ERGIC, spontaneous activation within the ERGIC/COPI compartment, block of anterograde transport to the GN and cell surface, and retrograde transport of activated TrkAIII from the ERGIC back to the ER, with subsequent TrkAIII inactivation in a self-perpetuating recycling mechanism, and also TrkAIII recruitment to the centrosome; the ways TrkAIII influences oncogenicity via IP3K signaling, induction of an adapted survival ER-stress response and modulation of centrosome behavior, are also shown. c) Representation of TrkAIII intracellular trafficking in the presence of TrkA tyrosine kinase (tk) inhibitors, showing anterograde transport of inactive immature *de novo* synthesized TrkAIII from the ER to ERGIC, inhibition of spontaneous activation within the ERGIC/COPI compartment, prevention of retrograde transport from the ERGIC back to the ER, relieving chronic ER stress and reducing recruitment to the centrosome, combined with the promotion of anterograde transport of inactivated TrkAIII from the ERGIC to the GN, associated with TrkAIII maturation and subsequent proteasome degradation.

Spontaneous TrkAIII activation in NB cells was inhibited by Brefeldin A and did not associated with GN disruption. This differs to a report that the aberrant spontaneous activation of immature TrkA within the ERGIC is not inhibited by Brefeldin A and causes GN disruption [11], identifying an additional difference between the spontaneous intracellular activation of TrkA and TrkAIII. Furthermore, we identified retrograde transport of activated TrkAIII from the ERGIC/COP-I compartment back to the ER, rather than GN disruption, as the major mechanism preventing anterograde TrkAIII transport to the GN [7]. Although the underlying mechanism for retrograde TrkAIII transport from the ERGIC to ER remains to be elucidated, it was prevented by TrkA tyrosine kinase inhibitors and, therefore, dependent upon TrkAIII tyrosine kinase activity. Since, TrkAIII does not contain classical KDEL sequences, it is possible that activated TrkAIII may interact with KDEL cargos destined to return to the ER in retrograde transport COPI vesicles or, alternatively, activated TrkAIII may exhibit motor protein-driven microtubule minus-end directed retrograde transport analogous to that used by ligand-activated cell surface TrkA receptors for intracellular transport [12, 13] but miss-localized to the ERGIC-COPI compartment, resulting in retrograde transport back to the ER. Such a mechanism would also explain the recruitment of active TrkAIII to the centrosome, the organizing centre and origin of microtubule arrays [14, 15]. Our studies indicate that TrkAIII is inactivated upon returning to the ER, presumable by ER-associated PTPases, setting up a self-perpetuating re-cycling mechanism through which TrkAIII activation within ERGIC/COPI membranes stimulates retrograde transport of activated TrkAIII back to the ER, with subsequent TrkAIII inactivation within the ER facilitating TrkAIII export back to the ERGIC.

Spontaneous activation of TrkAIII within the ERGIC/COP-I compartment, as for TrkT3 activation within COPII membranes and immature TrkA activation within ERGIC membranes, results in post receptor signaling through IP3K but not Ras/MAPK [1, 10, and 11]. This contrasts with post receptor signaling from ligand-activated cell surface TrkA through Ras/MAPK, which in NB models results in tumor suppressing activity [1, 2]. It is likely therefore, that signaling through IP3K but not Ras/MAPK from miss-localized spontaneously-activated TrkAIII underpins the its diametrically opposite oncogenic activity by promoting pro-survival, pro-angiogenic and pro-stem cell patterns of gene expression in the absence of proliferation inhibition and differentiation, induced by Ras/MAPK. This most likely reflects the dissociation of mislocalized TrkAIII from components of the Ras/MAPK pathway, which are abundant in both GN and cell surface membranes [1, 14-16].

In addition to oncogenic signaling through IP3K, retrograde transport of TrkAIII from the ERGIC back to the ER causes chronic ER-stress, resulting in a protective partially activated ER-stress response that is characterized by ATF6 but not Ire1a/Xbp-1 activation, increased Grp78/Bip expression and attenuated responsiveness to acute ER-stress induction, characteristic of the survival adaptation to chronic stress within the ER [1, 16-18]. This protective response adds an "oncogenic non-addiction" perspective to the oncogenic activity of TrkAIII, which together with IP3K signaling and recruitment to the centrosome, characterize the three fundamental mechanisms through which TrkAIII exerts its effects upon tumor behavior. All three mechanisms depend upon spontaneous TrkAIII activation within the ERGIC/COPI compartment and retrograde TrkAIII transport from the ERGIC back to the ER and also to the centrosome. The TrkA tyrosine kinase inhibitors CEP-701, Go6976 and GW441756 can prevent all three of these mechanisms. Our observations support a growing body of evidence that mislocalized activation of oncogenic receptor tyrosine

kinases switches downstream signaling outcomes, and are summarized in figure 1.

Conflicting interests

The authors have declared that no conflict of interests exists.

Acknowledgements

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Abbreviations

TrkA: tropomyosin-related kinase A; NGF: nerve growth factor; NB neuroblastoma; ERGIC: endoplasmic reticulum-Golgi intermediate compartment; ER: endoplasmic reticulum; GN: Golgi Network; PTPase: protein tyrosine phosphatase; IP3K: inositol phosphate-3 kinase; ATF-6: activating transcription factor-6; XBP-1: X-box binding protein-1; Ire1a: inositol-requiring enzyme; MAPK: mitogen-activated protein kinase.

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