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Alternative TrkA Splicing
and Neuroblastoma

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1. Introduction

Neuroblastoma is one of the most frequent solid paediatric tumours of the nervous system, accounting for up to 10% of all paediatric tumours. The majority of NBs originate from a sympathoadrenal cell lineage of neural crest origin during sympathetic nervous system development, and represent a heterogeneous group of tumours that exhibit a high degree of genetic and biological variability, including not infrequent spontaneous regression or differentiation to ganglioneuroma (Evans, 2004; Nakagawara, 2004). A large percentage of NB patients present with stage 4 disease characterised by dissemination primarily to bone; bone marrow; lymph node; liver and skin sites, with metastatic bone disease carrying automatic stage 4 diagnosis and the poorest prognosis. A subset of stage 4 NBs that disseminate primarily to liver skin and bone marrow sites exhibit frequent spontaneous regression and are classified as stage 4S. Genetic alterations that associated with aggressive NB include: amplification of the proto-oncogenic transcription factor N-myc in up to 20% of all NBs and up to 40% of aggressive NB; gain of chromosome 17 and loss of distal material from the chromosomes 1p32-pter (minimal common region 1p36.2); 14p23-qter; 11q23 and 18, regions likely to contain oncosuppressors (Evans, 2004; Nakagawara, 2004; Jiang et al., 2011; Takita et al., 2000). Despite general improvements in therapy, the age of onset plus high frequency of post-therapeutic relapse have meant that survival rates in patients with NB remain poor, highlighting the need for a greater understanding of the molecular mechanisms involved in this tumor type and the translation of this information into novel therapies.

Receptor tyrosine kinases (RTKs) regulate cellular growth, differentiation and survival during development. In general, RTK function depends upon appropriate ligand binding, with inappropriate activation and temporary duration of activation regulated by molecular domain, glycosylation status; protein chaperones; phosphorylation status and associated protein tyrosine phosphatases. The deregulation of RTK function is involved in tumour pathology, with over 30 RTKs associated with different malignancies, and is associated with

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a change in any one or combination of RTK regulatory mechanisms. Direct oncogenic activation of RTKs has been shown to result from gene amplification; novel chimera formation; deletion and point-mutation; and also by alternative and/or aberrant splicing (Bennasruone et al., 2004).

Alternative splicing is a fundamental physiological mechanism for differential protein expression from a single gene through alternative exon usage, is largely responsible for the proteomic complexity of higher organisms (Modek & Lee, 2002) and is also involved in cancer at the level of both oncogene activation and oncosuppressor inactivation. RTK oncogenes reported to be activated by alternative or aberrant splicing include the EGF receptor, FGF receptor-1; insulin receptor and nerve growth factor (NGF) receptor tropomyosin related tyrosine kinase (Trk) A (Kalnina et al., 2005; Tacconelli et al., 2004).

TrkA is the preferred receptor for NGF, plays a critical role in sympathetic nervous system development and is essential for the formation, differentiation and survival of normal sympathetic neurons that originate from cells of neural crest origin (Bibel & Barde, 2000). Not surprisingly, since the majority of NBs arise from a sympathoadrenal cell lineage of neural crest origin they also exhibit varying degrees of TrkA gene expression (Nakagawara et al., 1992; Nakagawara & Koger, 2000; Nakagawara, 2001). Mutation-activated TrkA oncogenes, however, have not been reported in NB and a possible NB tumour-suppressor function for TrkA has been suggested by an inverse relationship between expression and NB stage. Indeed, TrkA expression is considered to be a marker of better prognosis, potentially involved in both spontaneous and therapy-induced NB regression (Nakagawara et al., 1992; Nakagawara & Koger, 2000; Nakagawara, 2001). This hypothesis is supported by reports of TrkA tumour-suppressing activity in NB models, characterised by growth inhibition; differentiation, apoptosis and the inhibition of angiogenesis (Tacconelli et al., 2004; Nakagawara, 2001; Matsushima & Bogenmann, 2000; Lavenius et al., 1995; Lucarelli et al., 1997; Eggert et al., 2002).

Recently, however, the concept that TrkA plays an exclusively tumour suppressing role in NB has been challenged by discovery of a novel stress-regulated alternative TrkAIII splice variant, expressed by advanced stage primary human NBs, which exhibits tumour promoting activity in NB models and oncogenic activity in NIH-3T3 fibroblasts (Tacconelli et al., 2004).

In this chapter, therefore, we review current concepts concerning alternative TrkA splicing in NB, through comparative analysis of alternative TrkA splice variant structure, spontaneous versus ligand-mediated activation, post receptor signalling, regulation of expression, and tumour suppressing versus oncogenic activity. We also discuss the potential prognostic value of assessing alternative TrkAIII splicing and therapeutic ways to reduce TrkAIII involvement in NB.

2. The TrkA proto-oncogene

Trk-A is a member of the tyrosine kinase neurotrophin receptor family that includes TrkB and TrkC and is the preferred receptor for NGF but also binds the neurotrophin NT-3 (Klein et al., 1991; Patapoutian & Reichardt, 2001). The 25kb human TrkA gene is organised into 17 exons and maps to chromosome 1q21-q22 (Greco et al., 1996; Weier et al., 1995). Identification of the TrkA proto-oncogene was preceded by the discovery of the first tumour-associated TrkA oncogene (Klein et al., 1991; Martin-Zanca et al., 1986, 1989).

TrkA expression is absolutely required for the development, maturation and maintenance of the central and peripheral nervous systems. Within the peripheral nervous system TrkA regulates the formation, differentiation and survival of sympathetic neurones that originate from progenitors of neural crest origin (Bibel & Barde, 2000; Kaplan & Miller, 2000; Oppenheim, 1991; Ernsberger, 2009). TrkA is also critical for the development and function of the immune system (Vega et al., 2003; Fiore et al., 2009).
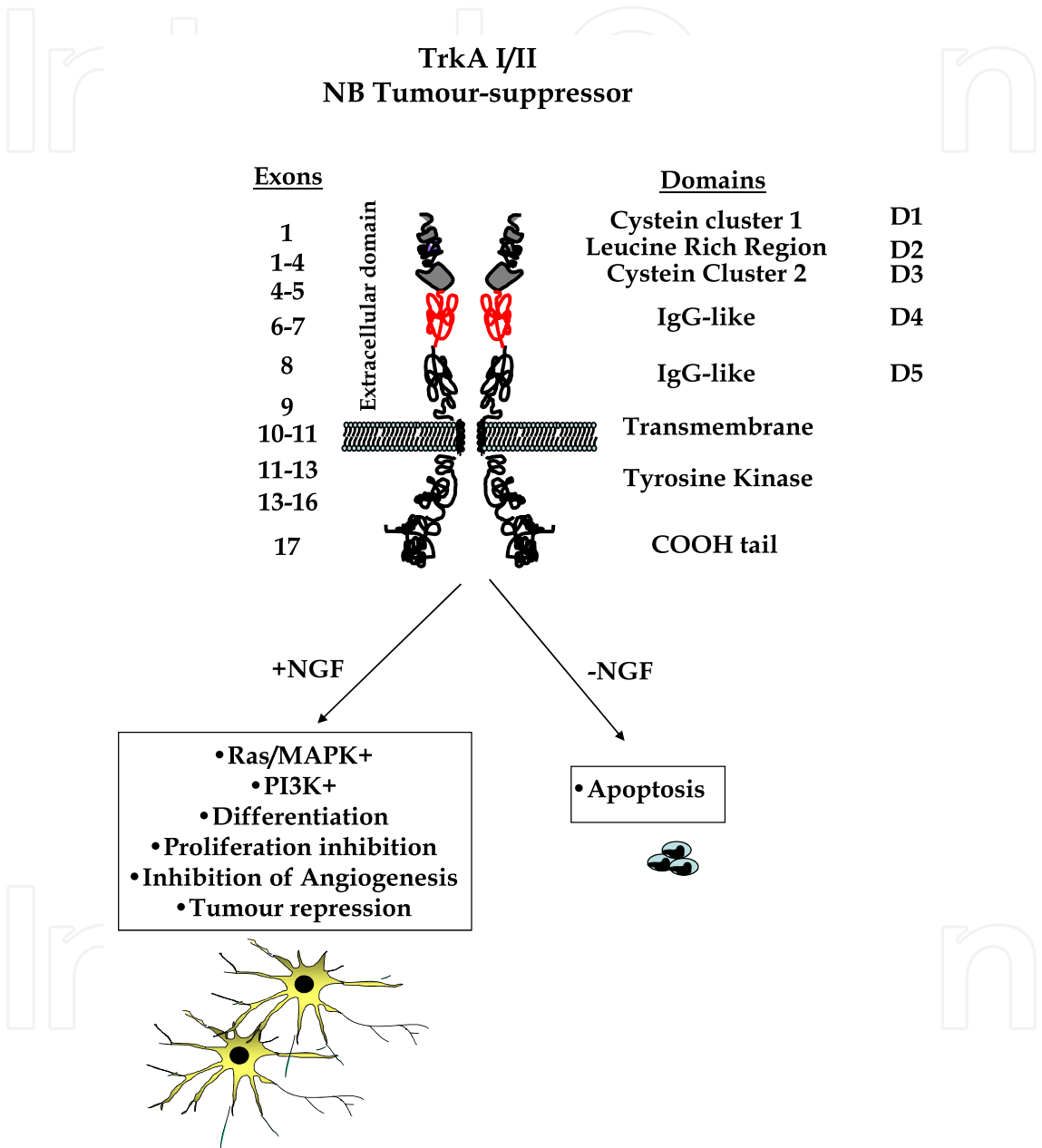
Representation of the exon and domain structure of TrkAI/II receptors and the biological effects of TrkA upon NB cell behaviour.

Fig. 1. The TrkAI/II NB tumour suppressor

The TrkA protein is expressed as a predominant 140kDa cell surface transmembrane tyrosine kinase receptor, comprised of an extracellular domain containing two cysteine-rich clusters (D1 and D3) interrupted by a leucine rich domain (D2) and 2 immunoglobulin (Ig)-

like domains (D4 and D5) involved in ligand-binding. Receptor transmembrane and juxta-membrane regions are critical for signal internalisation and transduction, whereas the intracellular tyrosine-rich carboxyl-terminal cytoplasmic domain exhibits tyrosine kinase activity upon ligand-mediated activation and is responsible for propagating post-receptor signal transduction (Windisch et al., 1995; Arevalo et al., 2000; Peng et al., 1995; Monshipouri et al., 2000; Kaplan et al., 1991; Holden et al., 1997; Wiesman et al., 2000) (Figure 1).

In the absence of ligand, cell surface TrkA receptors are maintained as inactive oligomers (Mischel et al., 2002) that concentrate within caveolin-1 and cholesterol-containing cell membrane caveolae invaginations, which also contain components of the Ras signalling pathway (Paratcha & Ibanez, 2002). In the absence of ligand, receptor oligomers are maintained in an inactive state by a mature extracellular domain N-glycosylation status, the presence of intact D4 and D5 domains and by receptor-associated protein tyrosine phosphatases (PTPases) (Arevalo et al., 2000; Marsh et al., 2003; Watson et al., 1999; Ostman & Bohmer, 2001; Sastry & Elferink, 2011). Upon ligand binding, oligomeric TrkA receptors alter conformation and acquire tyrosine kinase activity, which is facilitated by temporary inactivation of receptor-associated PTPases. This results in auto- and trans-phosphorylation of receptor tyrosine residues Y490, Y674/675, Y751 and Y785, which act as phosphorylation-dependent binding sites for a variety of signalling proteins. The adapters Shc and FRS-2 bind to phosphorylated Y490; Grb-2 and SOS bind to phosphorylated Y674 and Y675; the IP3K p85α subunit binds phosphorylated Y751 and PLCγ binds phosphorylated Y785. These interactions provide avenues for signal transduction through the Ras/MAPK, PI3K/Akt/NF-κB and PKC pathways, which mediate the effects of NGF upon cell differentiation, proliferation, survival and apoptosis (Kaplan & Stevens, 1994; Green & Kaplan, 1995; Hallberg et al., 1998; Meakin et al., 1999; Cunningham et al., 1997; Obermeier et al., 1993, 1994; Segal et al., 1996; Yao & Cooper, 1995). Neurotrophin activity is further modulated by interaction between TrkA and the low affinity p75[NTR] receptor (Peng et al., 1995; Hempstead et al., 1991; Majdan et al., 2001; Zaccaro et al., 2002; Nykjaer et al., 2005).

An additional feature of NGF/TrkA receptors is retrograde transport signalling within the cell. This depends upon receptor/ligand interaction, internalisation and retrograde transport of NGF-activated TrkA receptors, resulting in signal transduction within the cell body. Sympathetic neurons most dramatically illustrate this activity, with retrograde transport of NGF-activated TrkA along the axonal length to the neuronal cell body. This phenomenon appears to involve ubiquitin-mediated receptor internalisation through interaction with p75[NTR] and TRAF6, receptor endocytosis within clatherin coated vesicles and receptor endocytosis facilitated by the endocytosis inducing protein EHD4/Pincher (Moises et al., 2007; Howe et al., 2004; Valdez et al., 2005).

In addition to a cell surface localisation, immature forms of the TrkA receptor also localise to intracellular membranes of the Golgi Network (GN), where they can be trans-activated by agonists of the G-protein linked A$_{2A}$ adenosine receptor, providing evidence for intracellular neurotrophin-independent TrkA activation. Furthermore, post-receptor signal transduction from GN-associated TrkA differs from that of NGF-activated cell surface TrkA by signalling through PI3K/Akt but not Ras/MAPK, inducing NF-κB transcription factor activity and a more stress-resistant phenotype. TrkA localisation to the GN may not only reflect the transient passage of de-novo synthesised receptors but also alterations in receptor

extracellular domain N-glycosylation and/or folding (Watson et al., 1999; Rajagopal et al., 2004).

2.1 TrkA oncogenes

The first TrkA oncogene was identified in colon cancer as a novel constitutively active cytoplasmic chimera bearing tropomyosin substitution of the TrkA extracellular domain (Martin-Zanca et al., 1986, 1989). Subsequently, Trk-T1, Trk-T2 and Trk-T3 oncogenes were identified in papillary thyroid and colon tumours and characterised as constitutively active non-cell surface chimeric oncogenes. Trk-T1 and Trk-T2 oncogenes bear different extracellular domain substitutions with tropomyosin and Trk-T3 bears an extracellular substitution with Trk-fused gene, located on chromosome 3q11-12 (Coulier et al., 1990; Greco et al., 2010). Non-chimeric TrkA oncogenes include a constitutively active extracellular domain point mutated TrkA oncogene identified in prostate cancer (George et al., 1998) and a TrkA oncogene bearing an extracellular deletion of the D5 Ig-like domain identified in acute myeloid leukaemia (Reuther et al., 2000). Under experimental conditions TrkA oncogenes have been generated by: C345S and P203A point mutations within the receptor extracellular domain; by in-frame deletion of extracellular domain sequences encoding the Ig-like domains D4 and D5; and by duplication of the TrkA tyrosine kinase domain (Arevalo et al., 2000; Coulier et al., 1990).

In general, activated TrkA oncogenes are expressed as constitutively phosphorylated receptors, which exhibit spontaneous ligand-independent tyrosine kinase activity and signal chronically through PI3K/Akt/NF-κB, PKC and/or Ras/MAPK pathways, resulting in cellular transformation associated with alterations in proliferation and survival (Nakagawara, 2001; Martin-Zanca et al, 1989; Arevalo et al., 2000, 2001; Watson et al., 1999; Meakin et al., 1999; Coulier et al., 1990; Greco et al., 2010). Therefore in non-mutated TrkA, an intact fully N-glycosylated extracellular domain is critical for preventing ligand-independent receptor oligomerisation and spontaneous oncogenic activation, with essential roles played by extracellular domain P203 and C345 residues and by the extracellular D4 and D5 Ig-like domains (Arevalo et al., 2000, 2001; Watson et al., 1999; Coulier et al., 1990).

2.2 TrkA as an NB tumour-suppressor

NB arises from cells of neural crest origin during sympathetic nervous system development (Evans, 2004; Nakagawara, 2004; Jiang et al., 2011) and as a consequence exhibit varying degrees of TrkA expression (Tacconelli et al., 2004; Nakagawara et al., 1992; Nakagawara & Koger, 2000; Nakagawara, 2001). However, despite the absolute requirement for TrkA in the formation, differentiation and maintenance of the sympathetic nervous system; variable TrkA expression and the presence of non-coding TrkA gene polymorphisms and mutations in NBs (Scaruffi et al., 1999), there are no reports that associate mutation-activated TrkA oncogenes with this tumour type. On the contrary, TrkA is considered to be a potential NB tumour-suppressor. This concept is derived from the association between high level TrkA expression and low stage NB and has led to TrkA expression being considered a marker of better prognosis, and to the suggestion that TrkA expression may be required for both spontaneous and therapy-induced NB regression (Nakagawara et al., 1992; Nakagawara & Koger, 2000; Nakagawara, 2001; Brodeur et al., 2009; Tanaka et al., 1998; Suzuki et al., 1993). This hypothesis is supported by evidence that NB cells exhibit multiple defects in NGF receptor signalling (Azar et al., 1990); reports that NB differentiating agents such as retinoic acid and γ-interferon augment TrkA expression (Sugimoto et al., 2001), and reports that

TrkA gene transduction restores NGF-responsiveness to NB cells, inducing ligand-dependent signalling through PI3K, Ras/MAPK and PKC pathways, resulting in growth arrest, differentiation and the inhibition of angiogenesis and NB tumour xenograft growth (Tacconelli et al., 2004; Matsushima et al., 1990; Lavenius et al., 1995; Lucarelli et al., 1997; Eggert et al., 2002). A potential NB tumour-suppressor function for TrkA suggests that reduced TrkA expression would facilitate tumour progression. This is supported by the inverse relationship exhibited by TrkA expression and NB stage (Nakagawara et al., 1992; Nakagawara & Koger, 2000; Nakagawara, 2001) and suggests that the reintroduction of adequate TrkA expression levels and/or the activation of post cell surface TrkA receptor signalling represents an important potential therapeutic goal in NB.

Recently, however, a darker side to TrkA involvement in NB has been revealed by the discovery of an alternative TrkA splice variant "TrkAIII" expressed by advanced stage primary human NBs that exhibits oncogenic activity in NB models (Tacconelli et al., 2004; Farina et al., 2009a, 2009b).

3. Alternative TrkA splice variants

The human 25kb TrkA gene localises to chromosome 1p21-22, is organised into 17 exons (Greco et al., 1996; Weier et al., 1995) and exhibits alternative splicing. Alternative TrkA splice variants include: TrkA L0 and L1, which exhibit differential exons 2-4 usage (Dubus et al., 2000); TrkAI and TrkAII, which exhibit differential exon 9 use (Barker et al., 1993); and TrkAIII, which exhibits inframe skipping of exons 6 and 7, combined with exon 9 omission (Tacconelli et al., 2004).

3.1 TrkA L0 and L1

Alternative use of TrkA exons 2, 3 and 4 leads to the expression of alternative TrkA L0 (exons 2, 3 and 4 alternatively spliced) and L1 (exons 2 and 3 alternatively spliced) splice variants, originally described during rat development (Dubus et al., 2000). These variants would be expected to yield truncated receptors bearing inframe deletions of leucine-rich sequences encoded within exons 2-4 (Greco et al., 1996). Since, TrkA leucine rich motifs modulate ligand-binding to TrkA Ig-like domains (Windisch et al., 1995), L1 and L0 variants may exhibit altered ligand-binding activity, similar to that described for analogous alternative TrkB splice variants (Ninkina et al., 1997). Within the developing rat, the TrkA L1 variant is expressed within the thymus, testis, lung and kidney (Dubus et al., 2000). There are no reports that directly link TrkAL0 and L1 splice variants to tumour pathology. However, an uncharacterised 80kDa truncated TrkA isoform has been reported in human thymomas but it remains to be elucidated whether this represents an alternative splice variant, deletion mutant or degradation product (Parrens et al., 1998).

3.2 TrkAI and TrkAII

The differential use of exon 9, which encodes the amino acid sequence VSFSPV, results in the expression of alternative TrkAI (exon 9 exclusion) and TrkAII (exon 9 inclusion) splice variants (Barker et al., 1993). Both TrkAI and TrkAII variants are expressed as approximately 140kDa cell surface transmembrane receptors and the omission of exon 9 sequence does not result in ligand-independent receptor activation. Both variants bind NGF and NT3 neurotrophins (Barker et al., 1993; Clary et al., 1994) and exhibit a degree of tissue-specific expression, with TrkAII expressed predominantly by nervous system tissues and TrkAI expressed predominantly in thymic tissues (Barker et al., 1993). At the functional

level, TrkAII exhibits a higher level of NT3-mediated activation when co-expressed with the low affinity neurotrophin receptor p75**NTR** (Clary et al., 1994), which promotes apoptosis in the absence of TrkA signalling and regulates both ligand-dependent and independent TrkA activation to augment cell survival (Peng et al., 1995; Hempstead et al., 1991; Majdan et al., 2001; Zaccaro et al., 2002; Nykjaer et al., 2005). There are no other reported functional differences between the alternative TrkAI and TrkAII splice variants, suggesting that both isoforms are likely to be involved in nervous and immunological system development, maturation and maintenance through the regulation of proliferation, differentiation, apoptosis and survival, with potential for tissue-specific functional differences. There are no reports linking alternative TrkAI and TrkAII splicing to NB, suggesting that combined expression may represent a marker of better prognosis, inversely correlated with malignant NB behaviour (Tacconelli et al., 2004; Nakagawara et al., 1992; Nakagawara & Koger, 2000; Nakagawara, 2001, Brodeur et al., 2009; Tanaka et al., 1998; Suzuki et al., 1993; Cao et al., 2010).
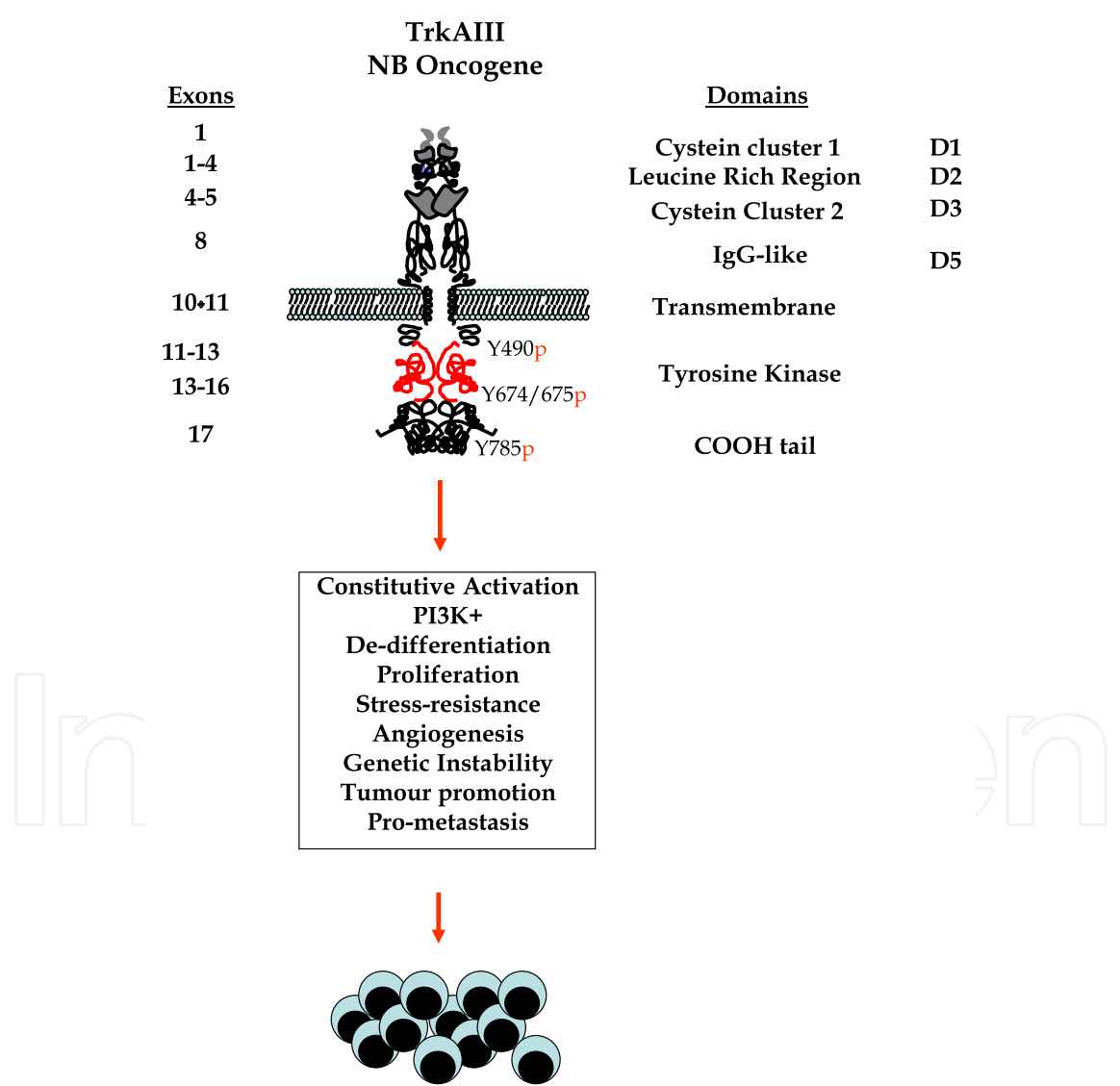
Representation of the exons and domain-structure of the TrkAIII receptor and its biological effects upon NB cells.

Fig. 2. The TrkAIII oncogene

3.3 The alternative TrkAIII splice variant

TrkAIII was originally identified as an unexpected RT-PCR product in mRNAs purified from primary human NBs and was subsequently cloned from the human SH-SY5Y NB cell line. TrkAIII was characterised as a novel alternative splice variant, exhibiting inframe skipping of exons 6 and 7, in addition to exon 9 omission (Figure 2) (Tacconelli et al., 2004). The skipping of exon 6 and 7 results in an in-frame deletion of amino acids 192-284, which encode the entire extracellular D4 Ig-like domain; introduces a valine substitution at the novel exon 5/8 splice junction; and causes the loss of several functional N-glycosylation sites (Tacconelli et al., 2004).

3.3.1 TrkAIII expression by primary NBs and NB cell lines

Originally identified in an advanced stage 4 primary human NB, preliminary data in 24 primary human NBs indicated predominant TrkAIII expression with respect to TrkAI/II in advanced stage 3 and 4 disease (Tacconelli et al., 2004). This has been recently confirmed in an independent study of 39 primary human NBs, in which a significant relationship between high TrkAIII expression and high stage was reported (Cao et al., 2010).

TrkAIII is expressed by human SK-N-SH, SH-SY5Y IMR32, SK-N-AS, KCNR, LAN5 and SK-N-BE NB cell lines, normal human neural stem cells and neural crest progenitors but not by differentiation-committed counterparts, (i.e cerebral granule neurones) (Tacconelli et al., 2004). This suggests a tissue-specific rather than NB tumour-specific alternative splice mechanism that potentially relates to an undifferentiated neural progenitor/stem cell-like tumour cell phenotype. TrkAIII expression by NB cells does not associate with mutations or deletions within the TrkA gene, supporting its status as an alternative splice variant (Tacconelli et al., 2004).

3.3.2 Epigenetic regulation of alternative TrkAIII splicing

The association between a high TrkAI/II to TrkAIII expression ratio with low stage NB and a low TrkAI/II to TrkAIII expression ratio with high stage NB (Tacconelli et al., 2004; Cao et al., 2010), supports the hypothesis that NBs switch from TrkAI to TrkAIII expression during progression. Alternative TrkAIII splicing may, therefore, represent a regulated tumour promoting switch, through which NB tumour suppressing signals from TrkAI/II can be converted to oncogenic signals from TrkAIII. Although the mechanisms involved in alternative TrkAIII splicing remain to be fully elucidated, potential epigenetic regulation of alternative TrkAIII splicing within the hostile tumour microenvironment is supported by the promotion of alternative TrkAIII splicing in human NB cell lines by conditions that mimic tumour-associated hypoxia (Tacconelli et al., 2004). The same conditions also promote alternative TrkAIII splicing in normal neural stem cells and undifferentiated neural crest progenitors, but not in differentiation committed counterparts (Tacconelli et al., 2004), suggesting that hypoxia-regulated alternative TrkAIII splicing in NB cells may represent conservation and pathological subversion of a physiological neural stem/progenitor cell hypoxia-protection mechanism. We do not exclude, however, that alternative and/or additional mechanisms may promote alternative TrkAIII splicing in NBs.

3.3.3 Differences between TrkAI/II and TrkAIII splice variants

Comparative analysis of TrkAI and TrkAIII receptors expressed in human SH-SY5Y NB cells has revealed several differences (Table 1). These include: a) Differences in molecular size.

TrkAI is expressed as immature 110kDa and mature gp140kDa N-glycosylated proteins that reduce to a single 80kDa protein upon tunicamycin treatment, whereas TrkAIII is expressed as a single 100kDa N-glycosylated protein that reduces to 70kDa upon tunicamycin treatment; b) Differences in receptor compartmentalization. The mature gp140 TrkAI receptor is expressed predominantly at the cell surface with GN accumulation of the immature gp110TrkAI receptor, whereas TrkAIII is not expressed at the cell surface but is retained within intracellular membranes, within which it exhibits relatively equal distribution between the endoplasmic reticulum (ER), endoplasmic reticulum and Golgi intermediate (ERGIC); Golgi network (GN) and associated vesicle compartments (Tacconelli et al., 2004; Farina et al., 2009a, 2009b); c) Differences in spontaneous versus ligand-dependent activation. TrkAI exhibits ligand-dependent but not spontaneous activity, whereas TrkAIII exhibits spontaneous ligand-independent activation and does not bind neurotrophins; d) Differences in post receptor signal transduction. In response to ligand, activated TrkA receptors exhibit receptor-associated tyrosine kinase and PI3K activity, are phosphorylated on Y490, Y674/675 and Y758 residues, bind Shc, Grb2 and FRS2 adapters and signal through PI3K/Akt and Ras/MAPK, whereas spontaneously active TrkAIII exhibits tyrosine kinase and PI3K activity, is constitutively phosphorylated on Y490, Y674/675 and Y758 residues, binds only low levels of non-phosphorylated Shc, does not bind FRS2 or GRB2 and signals through IP3K/Akt but not Ras/MAPK (Tacconelli et al., 2004); and e) differences in biological activity, with TrkAI exhibiting tumour-suppressing and TrkAIII oncogenic activity in NB models (Tacconelli et al., 2004).

These differences depend upon the omission of sequences encoded within exons 6/7 and form the basis of the differential TrkAI/II tumour-suppressing and TrkAIII oncogenic activity observed (Tacconelli et al., 2004; Farina et al., 2009a, 2009b). Intracellular TrkAIII retention versus cell surface TrkAI expression may depend upon differences in extracellular domain N-glycosylation, which regulates cell surface TrkA expression (Watson et al., 1999). Spontaneous ligand-independent TrkAIII activation most likely depends upon the omission of the extracellular D4 Ig-like domain plus associated N-glycosylation sites, which prevent ligand-independent TrkA activation (Arevalo et al., 2000). Differences in adapter protein binding exhibited by ligand-activated TrkAI and spontaneously-active TrkAIII and subsequent differences in post receptor signalling most likely reflect differences in receptor localisation, with lack of post TrkAIII signalling through Ras/MAPK explained either by dislocation from caveolae-associated Ras/MAPK (Paratcha & Ibanez, 2002; Rajagopal et al., 2004), altered adapter protein binding (Tacconelli et al., 2004; Farina et al., 2009a, 2009b); TrkAIII activity below the Ras/MAPK activation threshold (Hallberg et al., 1998); and/or direct PI3K/Akt inhibition of Raf-MEK-ERK signalling (Moelling et al., 2002), the latter possibility supported by TrkAIII antagonism of NGF/TrkAI-induced ERK phosphorylation (Tacconelli et al., 2004).

Interestingly, post TrkAIII receptor signalling through PI3K/Akt but not Ras/MAPK resembles post receptor signalling from GN-associated immature gp110TrkAI trans-activated by G-protein associated A_{2A} adenosine receptors (Rajagopal et al., 2004), indicating that an intracellular location alters post TrkA receptor signalling.

The characteristics exhibited by TrkAIII of spontaneous ligand-independent activity and chronic post receptor signalling are considered to be pre-requisites for oncogenic RTK activation (Bennasruone et al., 2004). How this relates to tumour suppressing signalling from ligand-activated cell surface TrkAI is dealt with below.

	TrkAI	TrkAIII
Expressed at the cell surface in caveolae	Yes	No
Spontaneous tyrosine kinase and PI3kinase activity	No	Yes
Constitutive tyrosine phosphorylation	No	Yes
Signals through Ras/MAPK upon activation	Yes	No
NB cell differentiation	Yes	No
Inhibits NB cell proliferation	Yes	No
Binds NGF	Yes	No
NB tumour suppressing activity	Yes	No
Oncogenic activity	No	Yes

Table 1. Summary of the differences between TrkAI and TrkAIII receptors expressed in SH-SY5Y NB cells.

3.3.4 TrkAIII as a NB oncogene

In contrast to the tumour-suppressing effects of TrkAI/II receptors in NB and NB models (Tacconelli et al., 2004; Nakagawara et al., 1992; Nakagawara & Koger, 2000; Nakagawara, 2001; Brodeur et al., 2006; Tanaka et al., 1998; Suzuki et al., 1993; Cao et al., 2010), TrkAIII transforms NIH3T3 fibroblasts rendering them tumourigenic in nude mice, promotes NB tumor-spheroid growth *in vitro;* and promotes primary (Tacconelli et al., 2004) and metastatic NB xenograft growth in nude mice (Figure 3), consistent with an oncogenic and pro-metastatic function. This is supported by the engineered deletion of the TrkA D4 Ig-like domain, which also results in oncogenic activation (Arevalo et al., 2000). TrkAIII, however, is also expressed by stressed non-tumourigenic normal neural stem cells, neural crest progenitors and normal thymocytes (Tacconelli et al., 2004, 2007), suggesting that TrkAIII transforming potential, like that of many oncogenes, depends upon additional factors.

A potential link between TrkAIII expression and metastatic bone disease, suggested by the increased capacity of TrkAIII transfected SH-SY5Y NB cells to form osteolytic bone metastases in nude mice (Figure 3), is also supported by the recent report that high TrkAIII expression and a low TrkAI/II to TrkAIII expression ratio associate significantly with metastatic bone disease in advanced stage human NB (Cao et al., 2010). The possibility that TrkAIII expression within primary tumours may both indicate and be involved in metastatic bone disease is of potential diagnostic/prognostic and therapeutic importance, considering that metastatic bone disease in NB carries immediate stage 4 diagnosis, very poor prognosis and is the major cause of post therapeutic disease relapse (Mugishima & Sakurai, 2000).

It is clear that the extracellular D4 domain and associated N-glycosylation sites, encoded within *TrkA* exons 6/7, are critical for correct TrkA receptor physiological function, cell surface expression, ligand-dependent activation and preventing ligand-independent receptor oncogenic activation (Arevalo et al., 2000; Watson et al., 1999). The loss of this domain confers oncogenic potential to TrkAIII (Tacconelli et al., 2004; Arevalo et al., 2000; Watson et al., 1999; Farina et al., 2009a, 2009b).
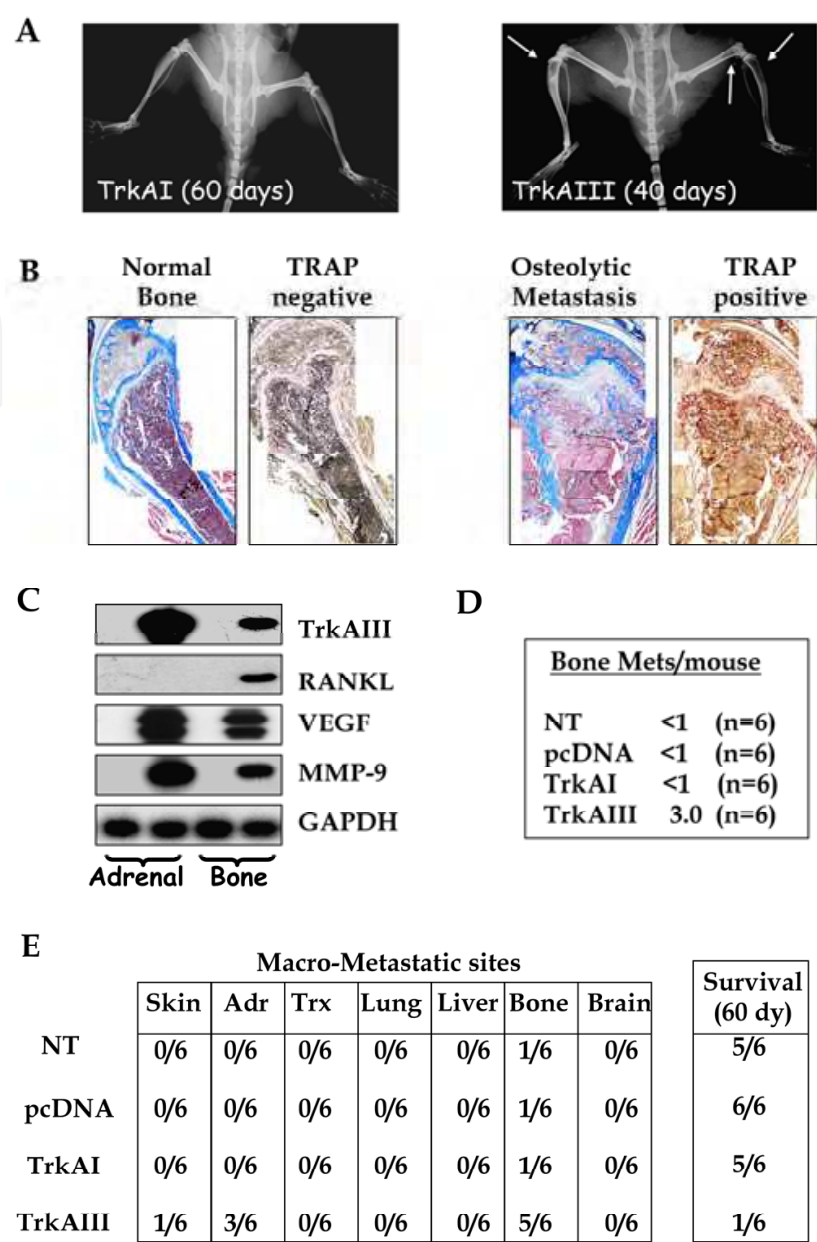
A) Radiographic appearance of normal bone and bone bearing osteolytic metastases (arrows) in representative nude mice injected via the intra-cardiac route with either TrkAI (60 days following injection) or TrkAIII (40 days following injection) transfected SH-SY5Y NB cells. B) Histological appearance of normal TRAP negative femur from a nude mouse 60 days following injection with TrkAI transfected SH-SY5Y cells and a TRAP positive osteolytic bone metastasis from a nude mouse 40 days following injection with TrkAIII transfected SH-SY5Y NB cells. C) RT-PCR Southern blot comparison of TrkAIII, RANKL, VEGF, MMP-9 and GAPDH mRNA expression in normal adrenal gland and normal bone versus adrenal and osteolytic bone metastasis from a nude mouse injected with TrkAIII expressing SH-SY5Y cells. D) The mean number of bone metastases per nude mouse 60 days following intra-cardiac injection with either non-transfected (NT), control pcDNA vector, TrkAI or TrkAIII transfected SH-SY5Y cells. E) The number and distribution of macro-metastases and 60 day survival-rate of nude mice injected via the intra-cardiac route with either non-transfected, empty pcDNA vector, TrkAI or TrkAIII transfected SH-SY5Y cells.

Fig. 3. TrkAIII Promotes osteolytic bone metastasis.

4. Getting to grips with intracellular TrkAIII

The intracellular membrane-associated retention of TrkAIII is a prerequisite for oncogenic potential and oncogenic activity in NB models. Therefore, a greater understanding of the intracellular membrane compartments within which TrkAIII locates and exhibits activity will improve our understanding of how TrkAIII exerts an oncogenic rather than tumour-suppressing function.

4.1. TrkAIII within the ER

Intracellular non-nuclear membranes are separated into the ER, ERGIC, GN, and associated transport vesicle compartments.

Ultracentrifugation fractionation of intracellular membranes, receptor pulse-labelling and indirect IF experiments clearly demonstrate that N-glycosylated immature gp110kDa TrkAI receptors move rapidly from the ER to the GN where they accumulate and mature into gp140kDa TrkA prior to being transported to the cell surface (Farina et al., 2009b). TrkAIII on the other hand, neither alters in molecular size nor reaches the cell surface but accumulates within the intracellular membrane compartment, exhibiting relatively equal steady-state distribution between ER, ERGIC, GN and vesicle membranes (Figure 4) (Farina et al., 2009a, 2009b).

TrkAIII accumulation within the ER suggests either that TrkAIII re-cycles back to the ER and/or exhibits difficulty overcoming the ER quality control (ERQC) system. The latter possibility is supported by constitutive association with between ER-associated TrkAIII and the ER chaperones Grp78/Bip and calnexin, which recognise and retain misfolded proteins within the ER as a protective measure (Gregerssen & Bross, 2010). In contrast, TrkAI interaction with these chaperones is not detected under normal conditions but is induced by conditions of ER-stress, which promote TrkAI ER-retention (Farina et al., 2009b). This implicates the TrkA D4 domain and associated N-glycosylation sites, omitted from TrkAIII, in the correct folding of TrkA within the ER and subsequent ER-exit of nascent TrkA receptors.

The possibility that TrkAIII ER-retention does not depend upon spontaneous receptor activation is supported by observations that TrkAIII is not activated throughout the ER ; that TrkAIII ER-retention is reduced by the PTPase inhibitor sodium orthovanadate in association with augmented TrkAIII activity (Figure 5) (Farina et al., 2009b), by the observation that tyrosine kinase dead Y674/675F mutated TrkAIII exhibits enhanced ER-retention and that inhibition of TrkAIII activity by the tyrosine kinase inhibitor CEP-701 promotes TrkAIII ER-retention (Farina et al., 2009a, 2009b). In contrast, cell surface gp140TrkAI but not intracellular GN-associated gp110TrkAI is activated by sodium orthovanadate. This suggests that intracellular TrkAI, unlike TrkAIII, is not maintained in an intracellular inactive state by associated PTPases (Figure 5). These observations indicate that TrkAIII within the ER exists largely in PTPase-inhibited form, complexed with Brp78/Bip and calnexin; with inactivation promoting ER-retention. The PTPases responsible for inhibiting TrkAIII within the ER remain to be elucidated.

### 4.2 TrkAIII within the ERGIC/GN/vesicle compartment

In SH-SY5Y NB cells, TrkAIII overcomes ERQC, confirmed by the loss of Grp78/Bip and calnexin binding by non-ER membrane-associated TrkAIII, and distributes to the ERGIC, GN and associated vesicles compartments in roughly equal steady-state levels (Farina et al., 2009a, 2009b). TrkAIII activation, on the other hand, exhibits a relatively restricted association with a peri-nuclear centralised vesicle population that closely overlaps ERGIC

and GN membranes (Farina et al., 2009a), suggesting conditions favourable for spontaneous TrkAIII activation localise to a relatively specific intracellular vesicle compartment in NB cells.

A

B
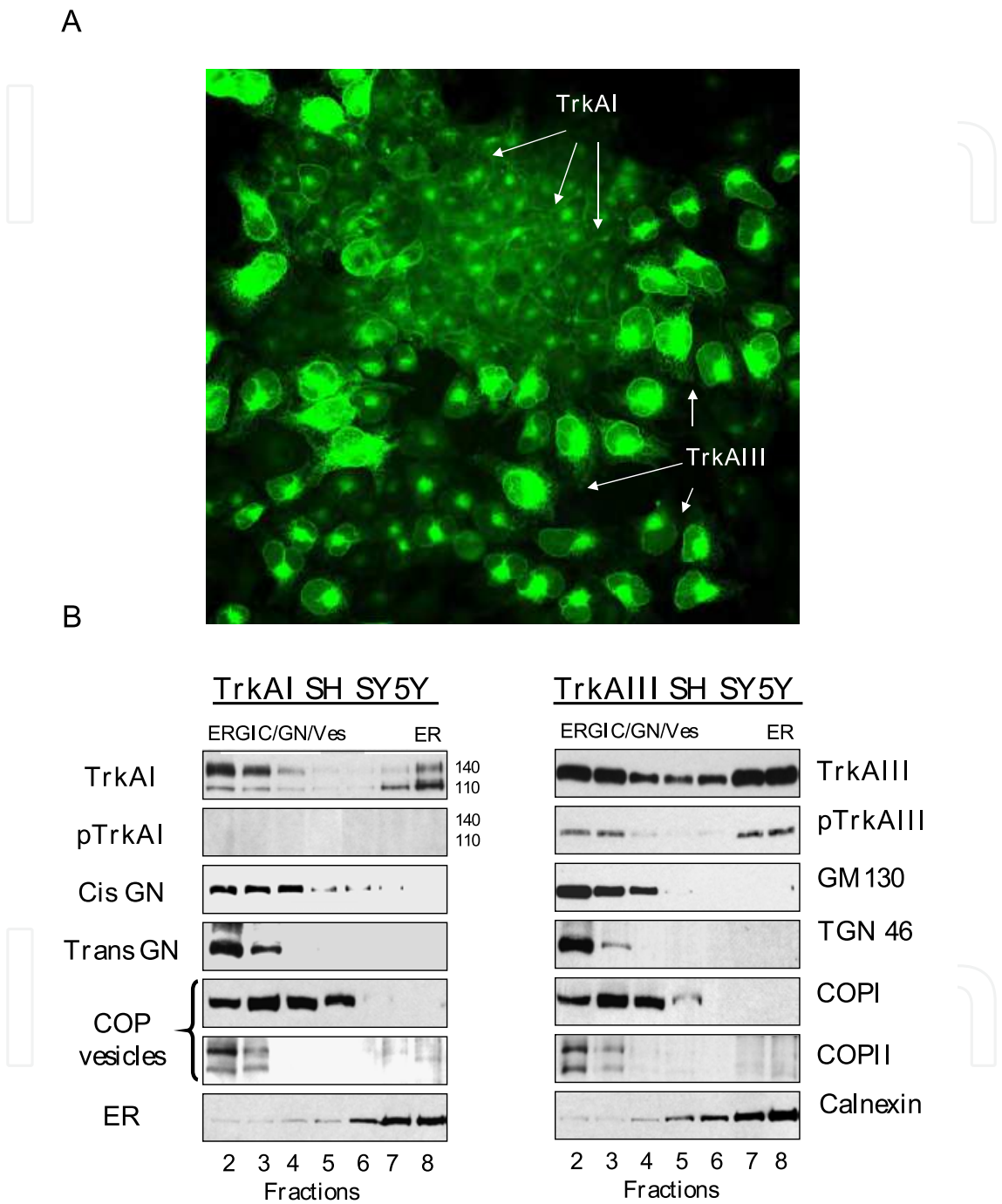
A) Indirect IF demonstrating differences in cell surface and GN-associated TrkAI expression and intracellular TrkAIII expression in a mixed population of TrkAI and TrkAIII transfected SH-SY5Y cells. B) Western blots demonstrating differences in TrkAI and TrkAIII distribution in ultracentrifugation density gradient-purified intracellular ER, GN and COP vesicle membranes from TrkAI and TrkAIII transfected SH-SY5Y cells.

Fig. 4. Comparison of TrkAI and TrkAIII localisation in SH-SY5Y cells.
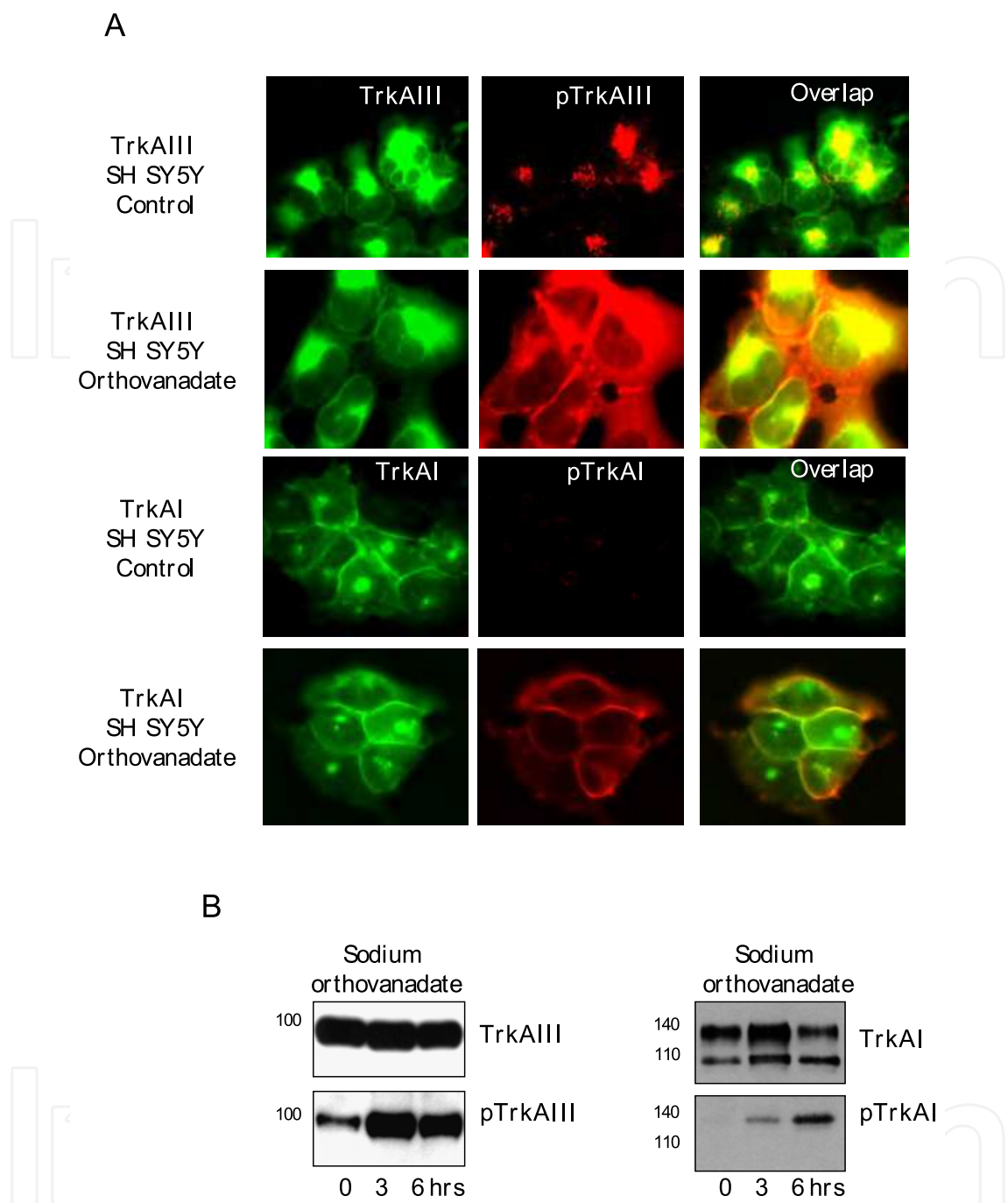
A) Indirect IF demonstrating the augmenting effect of the PTPase inhibitor sodium orthovanadate on intracellular TrkAIII tyrosine phosphorylation levels and capacity to induce tyrosine phosphorylation of cell surface gp140TrkAI but not intracellular gp110TrkAI, in stable transfected SH-SY5Y cells. B) Western blots demonstrating the augmenting effect of sodium orthovanadate upon total TrkAIII tyrosine phosphorylated levels and its capacity to induce tyrosine phosphorylation of TrkAI in stable transfected SH-SY5Y cells.

Fig. 5. Intracellular TrkAIII but not intracellular TrkAI exhibits spontaneous PTPase-regulated activity.

TrkAIII activation within this vesicle compartment is inhibited by Brefeldin A (Farina et al., 2009a, 2009b), a non-competitive inhibitor of G-protein the Arf-1 and ERGIC/GN disrupting

agent (Szul et al., 2007), indicating that TrkAIII activation in SH-SY5Y cells is ARF-1 dependent and occurs within the context of full ERGIC/GN assembly.

TrkAIII activation, furthermore, is not inhibited by anti-catalytic anti-NGF antibodies nor by c-Src inhibitors or A_{2A} adenosine receptor antagonists (Farina et al., 2009a, 2009b), confirming that TrkAIII activation in this vesicle compartment is spontaneous and ligand-independent, and most likely facilitated by D4 domain omission, combined with a localised deficit in PTPase activity.

Spontaneous TrkAIII activity within the ERGIC/GN associated vesicle compartment also depends upon TrkAIII interaction with the heat shock protein ATPase "Hsp-90", and is inhibited by the Hsp90 inhibitor Geldanamycin A (GA) and its clinically relevant analogues 17-AAG and 17-DMAG, currently in clinical trials for potential use in NB (Farina et al., 2009b; Szul et al., 2007). TrkAIII can, therefore, be added to EGFRvIII, BCR-Abl, Flt3 and PDGFR oncogene Hsp90 clients that exhibit sensitivity to GA and GA-analogues (Szul et al., 2007; Xu & Neckers, 2007). However, the potential therapeutic use of GA-analogues as TrkAIII inhibitors in NB is mitigated by the observations that GA and GA-analogues also inhibit TrkAI expression, neurotrophin-mediated TrkAI activation and subsequent post-receptor signalling, suggesting potential for side-effects on normal TrkA-dependent cellular functions. Furthermore, ER-associated inactivated TrkAIII induces a protective ER-stress response that is potentially involved in increasing NB cell resistance to GA-induced cytotoxicity (Farina et al., 2009b) (see section 7.3.).

Intracellular TrkAIII activation is also restricted to interphase, lost during mitosis in association with ERGIC/GN disruption, with re-activation observed post cytokinesis in association with ERGIC/GN reassembly (Farina et al., 2009a). TrkAIII activation is, therefore, subject to cell cycle regulation and occurs within the context of a fully assembled ERGIC/GN compartment.

Intracellular TrkAIII activated within ERGIC/GN-associated vesicles exhibits cytoplasmic tyrosine kinase domain orientation (Farina et al., 2009a). This places TrkAIII within a novel substrate context, a more detailed understanding of which will be critical for more detailed elucidation TrkAIII oncogenic function. This is exemplified by the novel interaction reported between TrkAIII and the interphase centrosome (Farina et al., 2009a) (see section 7.5.), which unveils a novel alternative oncogenic mechanism to "Classical" cell surface oncogenic RTK signalling.

5. Potential TrkAIII oncogenic mechanisms

5.1 Growth and differentiation

TrkA expression is considered to be a prerequisite for NB cell differentiation and tumour regression (Nakagawara, 2001). In support of this, TrkA gene transduction restores NGF responsiveness to NB cells and induces differentiation, growth arrest and/or apoptosis (Tacconelli et al., 2004; Matsushima et al., 1990; Lavenius et al., 1995; Lucarelli et al., 1997; Eggert et al., 2002). NGF/TrkA-mediated NB cell growth arrest and differentiation has been reported to depend upon Ras/MAPK pathway activation (Pumiglia & Decker, 1997). In contrast to TrkAI, TrkAIII transduction into NB cells neither induces growth arrest nor differentiation but inhibits the differentiation-inducing effects of NGF-activated TrkAI by antagonising Ras/MAPK signalling (Tacconelli et al., 2004). This places alternative TrkAIII splicing as a potential pivotal regulator of neurotrophin-mediated NB differentiation, helping to maintain NB cells in an undifferentiated state.

5.2 Angiogenesis

Angiogenesis is essential for primary and metastatic tumour growth and results in the formation of a relatively disorganised tumor neo-vasculature, required for oxygenation and nutrition of the growing tumour mass (Folkman, 2006). In contrast to TrkAI/II which has been reported to inhibit angiogenic factor expression and reduce tumour associated angiogenesis (Eggert et al., 2000, 2002), TrkAIII stimulates tumour-associated angiogenesis in NB models and alters the angiogenic equilibrium between MMP-9, VEGF and Tsp-1, increasing VEGF and MMP-9 but reducing Tsp-1 expression (Tacconelli et al., 2004). This equilibrium is an important determinant of tumour angiogenesis, since MMP-9 triggers VEGF-mediated angiogenesis and Tsp-1 inhibits both MMP-9 activation and VEGF activity (Bergers et al., 2000; Rodriguez-Manzaneque et al., 2001). The alteration of this equilibrium by TrkAIII is PI3K but not Ras/MAPK dependent (Tacconelli et al., 2004) and provides a potential angiogenic mechanism through which TrkAIII exerts oncogenic activity.

5.3 Induction of a more stress-resistant phenotype

The attainment of a more stress-resistant phenotype is not only involved in tumour progression but is a major determinant in eventual disease relapse from a "no evidence of disease" status. TrkAIII promotes a more stress-resistant NB cell phenotype, increasing resistance to the cytotoxic effects of the chemotherapeutic agents doxorubicin (Tacconelli et al., 2004) and GA (Farina et al., 2009b). This effect may not only involve protective PI3K/Akt/NF-κB signalling (Jaboin et al., 2002) but also the pre-conditioning of cells to stress as a result of partial activation of an ER-stress response (Farina et al., 2009b).

The ER stress response, reviewed elsewhere (Ron & Walter, 2007), is caused by the accumulation of unfolded or misfolded proteins within the ER, which results in the activation of three ER transmembrane proteins IRE1α, PERK and ATF6, all of which attempt to shift the ER back to homeostasis. PERK activation impedes protein translation, IRE1α activation splices XBP1 mRNA to produce the homeostatic transcription factor XBP1s which, together with activated ATF6(n), increases the transcription of genes that augment ER size, increase ER function and protect against apoptosis. Should this adaptive response be insufficient, the ER-response switches to apoptosis-inducing mode, considered to be caused by continuous IRE1α and PERK activation (Shore et al., 2011). In human SH-SY5Y NB cells, TrkAIII induces partial activation of the ER-stress response by activating ATF6(N) and increasing the expression of Grp78/BiP but does not induced XBP1 mRNA splicing to XBP1s (Farina et al., 2009b). This modified ER-stress response pre-conditions cells to resist further stress, helping to explain why TrkAIII expressing NB cells exhibit resistance to GA-induced cytotoxicity, despite GA-inhibition of TrkAIII tyrosine kinase activity (Farina et al., 2009b). This provides an additional oncogenic mechanism through which inactive TrkAIII retained within the ER may promote NB cell survival within the stressful tumour microenvironment.

5.4 Bone metastasis: stress resistance, angiogenesis and RANKL

TrkAIII expression in SH-SY5Y NB cells promotes the formation of osteolytic bone metastases in a nude mouse model. This effect is likely to involve the osteoclast-differentiation factor RANKL (Tanaka et al., 2005), the expression of which is induced by TrkAIII expressing NB cells within the bone metastatic environment, which together with a more angiogenic and stress-resistant phenotype results in the growth of osteolytic metastases (Figure 3). Furthermore, the relatively hypoxic bone environment would be

expected to promote alternative TrkAIII splicing in NB cells arriving within the bone marrow, providing a novel potential molecular mechanism for promoting NB metastasis to bone, consistent with Paget's "Seed and Soil" hypothesis for organ specific metastasis (Paget, 1989; Cao et al., 2010). This possibility is supported by the reported association between high level TrkAIII expression and low TrkAI/II to TrkAIII expression ratio with metastatic bone disease in NB patients (Cao et al., 2010).

5.5 Centrosome amplification and genetic instability

Interphase-restricted spontaneous activation of TrkAIII within ERGIC/GN-associated vesicles, in cytoplasmic tyrosine kinase domain orientation, results in interaction between a proportion of TrkAIII and the interphase centrosome, around which the ERGIC, GN and associated vesicle compartments assemble and integrate (Farina et al., 2009a; Mazzorana et al., 2011). This interaction is characterised by TrkAIII co-purification with centrosomes; TrkAIII binding of centrosome γ-tubulin; TrkAIII-mediated tyrosine phosphorylation of centrosome components; increased centrosome interaction with polo kinase 4; decreased centrosome interaction with separase; and centrosome amplification (Farina et al., 2009a). The result of this interaction is increased genetic instability characterised by: multi-polar spindle formation; mitotic catastrophe; anaphase DNA bridging; multinuclear cell formation; polyploidy and aneuploidy (Farina et al., 2009a). Therefore, TrkAIII also acts as a novel membrane-associated centrosome tyrosine kinase, promoting centrosome amplification and increasing genetic instability, unveiling an important and novel alternative mechanism through which TrkAIII exerts oncogenic activity.

TrkAIII promotion of genetic instability, combined with increased stress-resistance and angiogenesis, suggests that TrkAIII could act early during tumour progression to promote the accumulation of genetic damage within the hostile primary tumour microenvironment, progressing malignant tumours inevitably towards eventual oncosuppressor loss.

6. The clinical significance of assessing alternative TrkAIII splicing in NB prognosis

The possibility that alternative TrkA splicing represents a novel diagnostic/prognostic factor in NB is supported by two reports (Tacconelli et al., 2004; Cao et al., 2010). In the first, TrkAI/II and TrkAIII expression assessed by densitometric RT-PCR/Southern blotting in 24 primary human NBs found predominant TrkAIII over TrkAI/II expression in 1 of 11 stage 1/2 , and in 6 of 13 stage 3/4 NBs. A significantly higher TrkAI/II to TrkAIII expression ratio was also detected in stage 1/2 compared to stage 3/4 NBs, suggesting that the TrkAI/II to TrkAIII expression ratio decreases in association with disease progression and visa versa. In the second report (Cao et al., 2010), SYBR I Green fluorescent quantitative PCR was used to quantify alternative TrkAI/II and TrkAIII splicing in 39 NBs and results used to estimate 5 year survival rates. In this study, TrkAI/II expression was found to be significantly lower in tumours of high stage and higher in tumours of low stage, whereas TrkAIII expression was low in low stage tumours and significantly higher in high stage tumours. Univariate analysis revealed that the 5 year survival rate of patients aged >1year with abdominal tumors, bone metastases and a low TrkAI/II to TrkAIII ratio was significantly lower than controls, whereas multivariate analysis demonstrated that only a low TrkAI/II to TrkAIII ratio and bone metastasis exerted a negative impact upon the 5-year survival rate. From these studies, it appears that high TrkAI/II expression characterises NBs

with better prognosis, whereas high TrkAIII expression characterise NBs with poor prognosis, suggesting that early assessment of alternative TrkA splicing, combined with bone scanning, may represent an important diagnostic/prognostic parameter in NB.

7. Potential therapeutic ways of reducing TrkAIII involvement in NB

TrkAIII exhibits predominant expression in advanced stage NB and promotes oncogenesis in NB models in association with spontaneous activation and chronic post-receptor signalling through the PI3K/Akt/NF-κB but not Ras/MAPK pathway. This results in an impediment to NB cell differentiation and growth inhibition; augmented stress- resistance, increased genetic instability, and a more angiogenic and tumorigenic phenotype (Tacconelli et al., 2004; Farina et al., 2009a, 2009b). This suggests that inhibitors of TrkAIII tyrosine kinase activity and/or post TrkAIII signalling, may be an important addition to future NB therapy.

Within this context, the Trk kinase inhibitors K252α and its analogue CEP-701; the ARF inhibitor BFA and the Hsp90 inhibitor GA have all been shown to inhibit TrkAIII tyrosine kinase activity (Farina et al., 2009a, 2009b). CEP-701 reversibly inhibits TrkAIII tyrosine kinase activity and TrkAIII-induced centrosome amplification at nanomolar concentrations (Farina et al., 2009a), exhibits anti-tumor activity in NB xenograft models (Evans et al., 1999) and is in clinical trials in NB patients (Brodeur et al., 2009). The fungal macrolide BFA, a non-competitive interfacial inhibitor of the interaction between *sec7* domain-containing ARF-GEFs and ARFs 1 and 5, that disrupts the ERGIC/GN compartment by inhibiting the GDP-GTP ARF cycle necessary for vesicle assembly (Vigil et al., 2010; Pommier & Cherfils, 2005), reversibly inhibits TrkAIII tyrosine kinase activity in association with ERGIC/GN disruption (Farina et al., 2009a, 2009b). BFA exhibits anti-tumour activity that is mainly additive to standard chemotherapeutic agents in neuroendocrine tumor cells (Larsson et al., 2009), suggesting therapeutic potential for interfacial inhibitors of sec7 domain ARF-GEF/ARF interaction in reducing TrkAIII tyrosine kinase involvement in NB. The ansinomycin antibiotic, Hsp90 inhibitor, GA and its clinically relevant analogues, 17-AAG and 17-DMAG, inhibit RTK oncogene activity, NB xenograft tumour growth and are currently in clinical trials for potential future use in NB (Szul et al., 2007; Kang et al., 2006). GA and GA-analogues reversibly inhibit TrkAIII tyrosine kinase activity and inhibit proliferation of TrkAIII expressing NB cells (Farina et al., 2009a, 2009b), suggesting potential therapeutic use for reducing TrkAIII involvement in NB.

Inhibitors of TrkAIII tyrosine kinase activity, however, do not inhibit TrkAIII expression nor promote TrkAIII degradation but cause redistribution of inactive TrkAIII back to the ER, with potential to induce and/or augment a protective ER-stress response (Farina et al., 2009a, 2009b). This may explain the high level of resistance to GA-mediated cytotoxicity exhibited by TrkAIII but not TrkAI transfected NB cells, associated with an altered ER-stress response, despite GA-mediated inhibition of TrkAIII activity (Farina et al., 2009a, 2009b). Therefore, in addition to potential off-target effects, reversible TrkAIII-targeted kinase inhibitors may increase stress-resistance by promoting TrkAIII ER-retention and inducing a subsequent ER-stress response. Indeed, GA selects resistant slow growing TrkAIII expressing NB cells from mixed populations, which exhibit TrkAIII re-activation post GA removal, suggesting a potential mechanisms for post GA-therapy relapse (Farina et al., 2009b). We consider, therefore, that combined inhibition of TrkAIII expression and activity may represent a preferable therapeutic goal. For this purpose, we are currently developing specific peptide nucleic acid (PNA) inhibitors of TrkAIII expression based upon sequence at the novel

exon5/exon 8 splice junction (TrkAIII PNA conjugate (KKAA)$_4$-GGCCGGGACAC) (Farina et al., 2009a, 2009b). Current TrkAIII PNA conjugates inhibit TrkAIII but not TrkAI expression at micromolar concentrations and restore sensitivity to GA-induced cytotoxicity in TrkAIII expressing NB cells (Farina et al., 2009b). We are further developing these inhibitors in order to optimise uptake and lower effective inhibitory concentrations.
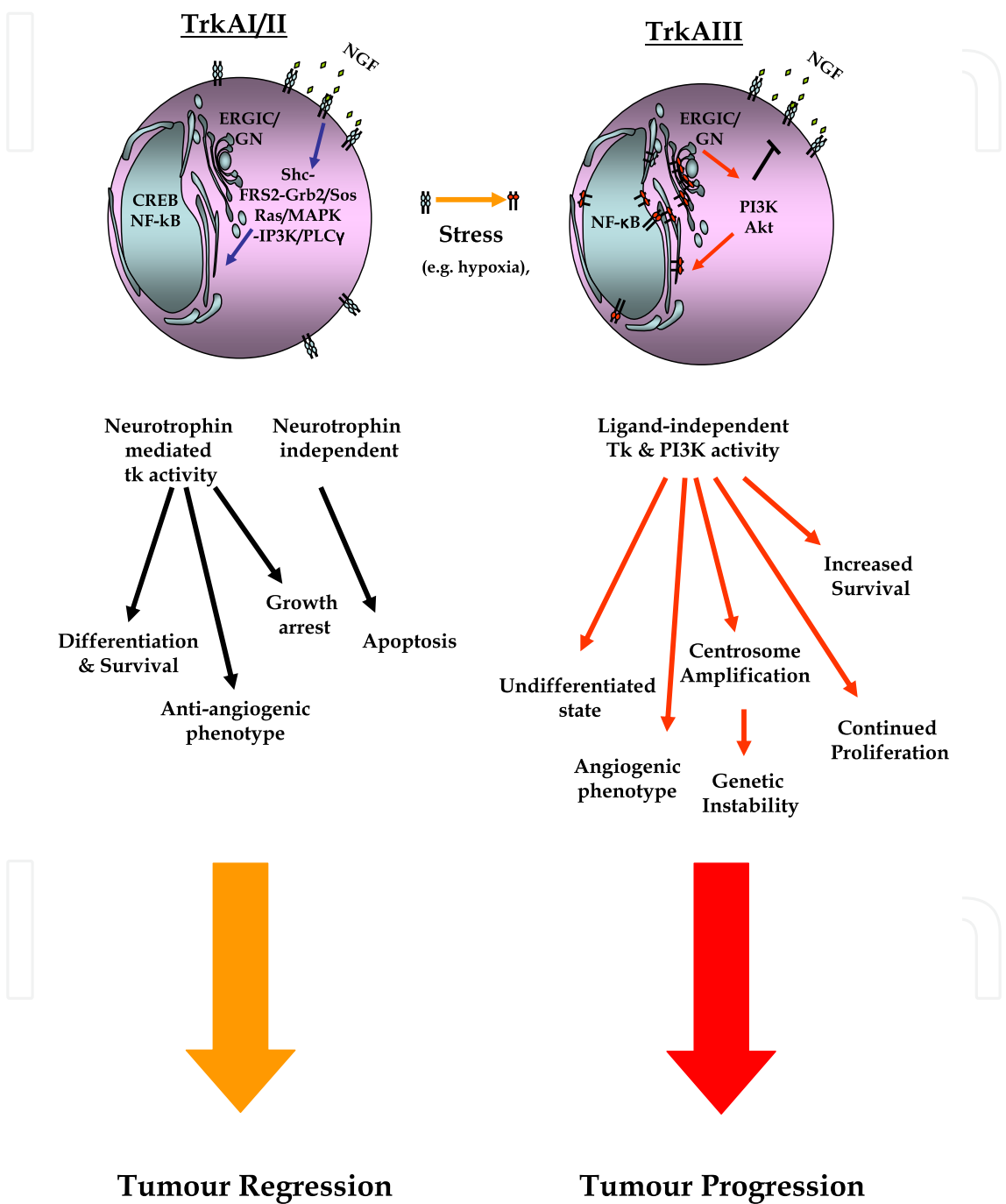
Summary of the differences in signalling, localisation and biological tumour suppressing outcome of TrkAI/II expression and tumour promoting/oncogenic outcome of stress-regulated alternative TrkAIII splicing

Fig. 6. Alternative TrkAIII splicing: a regulated NB tumour-promoting switch.

At the post receptor signalling level, PI3K/Akt pathway inhibitors reverse the pro-angiogenic effect of TrkAIII on the MMP-9/VEGF/TSP-1 equilibrium and may, therefore, be useful in reducing the pro-angiogenic TrkAIII effects, in addition to reducing protective PI3K/Akt/NF-κB signalling in TrkAIII expressing NB cells (Tacconelli et al., 2004).

The further elucidation of the molecular mechanisms responsible for promoting alternative TrkAIII splicing in NB cells may provide novel ways to reverse this process to favour tumour-suppressing TrkAI/II splicing.

8. Conclusions

The association between alternative TrkAIII splicing and advanced stage NB; the tumour-suppressing potential of TrkAI/II and oncogenic/tumour promoting potential of TrkAIII in NB models, and hypoxia up-regulation of alternative TrkAIII splicing in NB cells, suggest that stress-regulated alternative TrkAIII splicing may represent a pivotal promoter of NB tumour progression. The mechanisms through which intracellular TrkAIII exerts its oncogenic effects include: maintenance of a de-differentiated state; increased stress-resistance; increased genetic instability and the promotion of a more angiogenic and tumourigenic phenotype, and are summarised in Figure 6.

We propose that the assessment of alternative TrkA splicing in NB is of potential prognostic/diagnostic value and that the best way to limit TrkAIII and reduce its tumour promoting influence would be to combine inhibitors of TrkAIII expression (PNA and/or siRNA) and tyrosine kinase activity (CEP-701, BFA, GA-analogues), within the context of current chemotherapeutic protocols.

9. Acknowledgements

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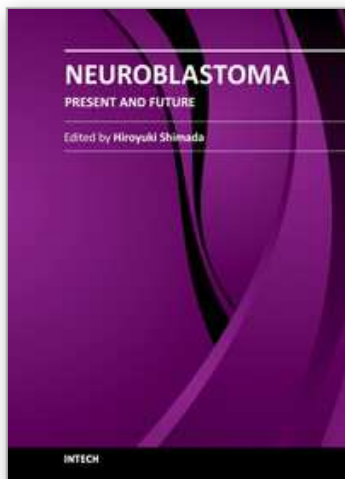
Neuroblastoma - Present and Future

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Neuroblastoma, once called "enigmatic", due to "unpredictable" clinical behaviors, is composed of biologically diverse tumors. Molecular/genomic properties unique to the individual tumors closely link to the clinical outcomes of patients. Establishing risk stratification models after analyzing biologic characteristics of each case has made a great success in patient management. However, the trend of improving survival rates in neuroblastoma over the last 30 years has started to level off, and currently available treatment modalities have almost reached to their maximized intensity. Furthermore, aggressive treatment causes significant long-term morbidities to the survivors. We really need to make the next step to the level of personalized medicine with more precise understanding of neuroblastoma biology. This book includes useful data and insights from the world's experts in this field. I believe this book can make an excellent contribution to all the investigators working hard and fighting for the children stricken by this disease.

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