

A tripartite complex composed of ETV6-NTRK3, IRS1 and IGF1R is required for ETV6-NTRK3-mediated membrane localization and transformation

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ETV6-NTRK3 (EN), a chimeric tyrosine kinase generated by t(12;15) translocations, is a dominantly acting oncoprotein in diverse tumor types. We previously showed that insulin-like growth factor 1 receptor (IGF1R) is essential for EN-mediated oncogenesis and that insulin receptor substrate 1 (IRS1) is constitutively tyrosine phosphorylated and bound by EN in transformed cells. Given that IRS1 is also an adapter for IGF1R, we hypothesized that IRS1 might localize EN to IGF1R at the membrane to activate phosphatidylinositol 3-kinase (PI3K)-Akt, which is critical for EN oncogenesis. In this study, we examined EN/IRS1/IGF1R complexes in detail. We find that both IRS1 and kinase active IGF1R are required for EN transformation, that tyrosine phosphorylated IRS1 is present in high molecular weight complexes with EN and IGF1R, and that EN colocalizes with IGF1R at the plasma membrane. Both IGF1R kinase activity and an intact cytoplasmic Y950 residue, the IRS1-docking site of IGF1R, are required, confirming the importance of the IGF1R/IRS1 interaction for EN oncogenesis. The dual specificity IGF1R and insulin receptor (INSR) inhibitor, BMS-536924, blocks EN transformation activity, cell survival and its interaction with IRS proteins, and induces a striking shift of EN proteins to smaller sized molecular complexes. We conclude that a tripartite complex of EN, IRS1 and IGF1R localizes EN to the membrane and that this is essential for EN-mediated transformation. These findings provide an explanation for the observed IGF1R dependency of EN transformation. Blocking IGF1R kinase activity may, therefore, provide a tractable therapeutic strategy for the many tumor types driven by the EN oncoprotein.

Keywords: ETV6-NTRK3; insulin-like growth factor 1 receptor (IGF1R); insulin receptor substrate 1 (IRS1); BMS-536924; chimeric tyrosine kinase

Introduction

The ETV6-NTRK3 (EN) chimeric oncoprotein is generated by t(12;15)(p13;q25) translocations in congenital fibrosarcoma (Knezevich *et al.*, 1998), cellular mesoblastic nephroma (Knezevich *et al.*, 1998; Argani *et al.*, 2000), adult acute myeloid leukemia (Eguchi *et al.*, 1999) and secretory breast carcinoma (Tognon *et al.*, 2002). More recently, ETV6-NTRK3 fusion transcripts were detected in mammary analog secretory carcinoma of salivary glands (Skalova *et al.*, 2010), secretory-like skin carcinoma (Kazakov *et al.*, 2010) and chronic eosinophilic leukemia (Forghieri *et al.*, 2011). EN transcripts encode the sterile α motif dimerization (SAM) domain of the ETV6 transcription factor linked to the tyrosine kinase domain of the neurotrophin-3 receptor NTRK3 (Knezevich *et al.*, 1998). EN expression leads to elevated expression of cyclin D1 mRNA and protein, which correlates with increased cell-cycle progression (Tognon *et al.*, 2001). EN expression in NIH3T3 fibroblasts constitutively activates two major effector pathways of *wt* NTRK3, namely the Ras-Erk and phosphatidylinositol 3-kinase (PI3K)-Akt cascades, and EN transformation is blocked by inhibition of either pathway (Tognon *et al.*, 2001). Wild-type NTRK3 utilizes the juxtamembrane residue Y516 to bind adapters such as Shc, SH2B or the PI3K p85 subunit to activate downstream signaling pathways (Huang and Reichardt, 2003). However, direct binding of EN to known NTRK3 adapters has not been demonstrable (Wai *et al.*, 2000; Lannon and Sorensen, 2005), likely because Tyr-516 is absent from EN due to the position of the t(12;15) fusion point (Wai *et al.*, 2000). Instead, EN directly binds the major insulin-like growth factor 1 receptor (IGF1R) substrate, insulin receptor substrate 1 (IRS1) through an NPXY motif in the EN C-terminus and the PTB domain of IRS1 (Lannon *et al.*, 2004).

IRS1 binds EN and is constitutively tyrosine phosphorylated in EN-transformed cells (Morrison *et al.*, 2002), and EN/IRS1 complexes recruit Grb2 and p85 to activate Ras-Erk and PI3K-Akt, respectively (Morrison *et al.*, 2002).

EN fails to transform mouse embryo fibroblasts derived from mice with a targeted disruption of the *Igf1r* gene (R⁻ cells), but reintroduction of IGF1R into R⁻ cells (R⁺ cells) restores EN transformation activity (Morrison *et al.*, 2002). IGF1R is essential for PI3K-Akt-dependent cell survival of EN-transformed cells, and both EN and IRS1 can be detected in membrane fractions of R⁺ cells but not of R⁻ cells (Martin *et al.*, 2006). A myristoylated form of EN (ENmyr) that is constitutively membrane-associated rescues EN transformation activity in R⁻ cells (Martin *et al.*, 2006). Taken together, these data point to an essential role for IGF1R-mediated membrane localization of EN for its transformation activity. Given that IGF1R binds IRS proteins through their SAIN domains (Craparo *et al.*, 1995), we postulated that IRS1 functions as a bridging molecule between EN and IGF1R to localize EN to the membrane where it can activate PI3K-Akt signaling. In the present work, we present evidence for an IGF1R/IRS1/EN complex, and demonstrate that disruption of either IGF1R kinase activity or IRS1-binding activity significantly impacts EN complex formation, localization, activation of the PI3K-Akt pathway and transformation activity. In addition, we use mass spectrometry to show that in HEK293 cells, EN binds two other IRS family members, IRS4 and IRS2, and that IGF1R kinase inhibition dramatically reduces these interactions. This work highlights the importance of a tripartite complex comprising EN, IRS proteins and IGF1R in EN-mediated transformation, and suggests a mechanism to explain the IGF1R dependency of EN oncogenesis.

Results and discussion

IRS1 and IGF1R kinase activity are required for EN transformation

IRS1 binds EN and is constitutively tyrosine phosphorylated in EN-transformed cells (Morrison *et al.*, 2002). To confirm an essential role for IRS1 in EN-mediated transformation, we used the IL-3-dependent murine myeloid 32D cell line that expresses IGF1R but lacks either IRS1 or IRS2 expression (Sun and Baserga, 2004). These cells fail to survive in media lacking IL-3 unless cells are engineered to overexpress and secrete IL-3. EN expression in 32D cells alone did not confer IL-3-independent growth or survival (Figure 1a, top and bottom) unless IL-3 was added to the medium (Supplementary Figures S1a and b). However, co-expression of IRS1 along with EN conferred both proliferation and survival properties to 32D cells in the absence of IL-3, confirming that EN requires IRS1 to transform cells (Figure 1a, top and bottom). Since IRS1 overexpression dramatically enhances the transforming ability of EN in NIH3T3

cells (*Igf1r*+/+) (Lannon *et al.*, 2004), we tested if IRS1 overexpression alone could support EN transformation. However, overexpression of IRS1 in *Igf1r* null R⁻ cells transduced with EN (Figure 1b, top) failed to overcome the IGF1R requirement for EN transformation (Figure 1b, bottom). These findings indicate that EN transformation requires both IGF1R and IRS1.

IRS1 binds to activated IGF1R through a phosphorylated cytoplasmic Y950 residue of the latter (Craparo *et al.*, 1995). To test whether this activity is necessary to support EN transformation, we generated *wt*, kinase dead (K1003A, or KA), and IRS1-binding defective Y950F (YF) mutants of the kinase-containing cytoplasmic β -subunit of IGF1R, but lacking the extracellular ligand binding domain. Each was tethered to the plasma membrane using a myristoylation tag (intracellular myristoylated, or ICmyr; Supplementary Figure S2). These constructs or a non-tethered *wt* intracellular IGF1R form lacking the myristoylation signal (designated IC) was expressed in *Igf1r* null R⁻ cells and detected using antibodies against the IGF1R β subunit (Figure 1c, top panel). As shown in Figure 1c (bottom panels), only mutants retaining the *wt* IGF1R kinase, IRS1-binding activity (that is, intact Y950) and the myristoylation tag could support EN activation of the critical transformation-associated PI3K-Akt pathway, as assessed by Akt and GSK3 β phosphorylation. Furthermore, only R⁺ cells, or R⁻ cells expressing ICmyr with intact kinase and IRS1-binding domains, exhibited EN-mediated soft agar colony formation (Figure 1d). Taken together, these results strongly indicate that EN transformation requires a kinase active, membrane tethered form of IGF1R that retains IRS1-binding activity.

EN interacts with phosphorylated IRS1 in high molecular weight complexes

We previously demonstrated that EN forms high molecular weight complexes and that this is essential for EN-mediated transformation (Tognon *et al.*, 2004). Moreover, when protein lysates from EN-expressing cells are subjected to fast protein liquid chromatography (FPLC), EN is observed in fractions ranging from 45 to $>2 \times 10^6$ kDa (Tognon *et al.*, 2004). To identify potential tyrosine phosphorylated proteins interacting with EN in high molecular weight fractions, we immunoprecipitated EN from different FPLC fractions and performed α -phosphotyrosine western blotting. Similar to previous results (Tognon *et al.*, 2004), tyrosine phosphorylated EN was detected in 150 to 1×10^6 kDa sized fractions (Figure 2a, top). Two additional major tyrosine phosphorylated proteins of ~ 200 and 180 kDa co-precipitated with EN, but only the 180-kDa species was present in high molecular weight fractions and not detected in lysates from vector control and kinase dead EN-K380N-expressing control cell (Supplementary Figure S3). Since IRS1 interacts with EN and is constitutively tyrosine phosphorylated in EN-transformed cells (Morrison *et al.*, 2002), we used α -IRS1 antibodies to reprobe the Figure 2a blot, confirming the 180-kDa band as IRS1 (Figure 2a,

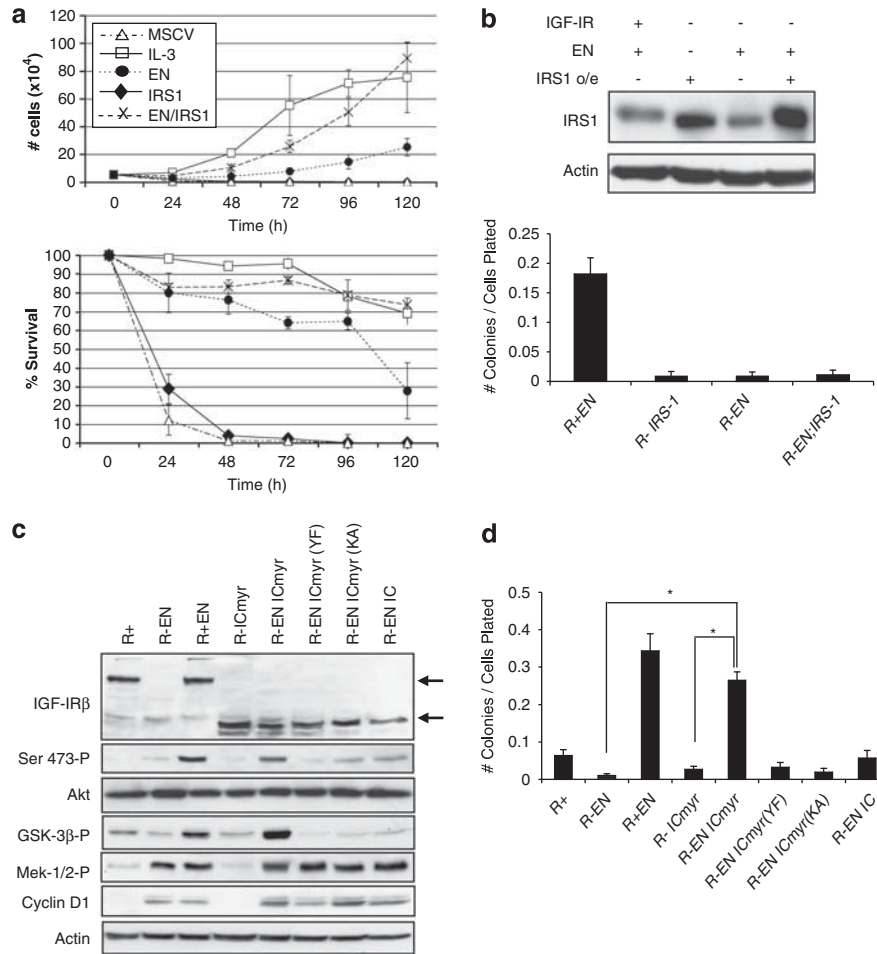


Figure 1 Both IRS1 and IGF1R are required for EN-mediated transformation. **(a)** Co-expression of both EN and IRS1 was required to promote IL-3-dependent growth (top) or survival (bottom) in IRS1-deficient 32D cells. Results represent an average \pm s.d. of three independent experiments. **(b)** Western blot analysis confirming the overexpression (o/e) of IRS1 in IGF1R null cells (R-) in the presence (+) or absence (-) of EN expression (top). Results of soft agar colony formation assays (bottom). Overexpression of IRS1 in EN cells cannot overcome the lack of IGF1R expression. The results are an average \pm s.d. of three independent experiments. **(c)** IGF1R(ICmyr) rescues Akt activation and transformation in R-EN fibroblasts. R+ (IGF1R positive), R-EN(IGF1R-/-), R+ EN, R-ICmyr (myr IGF1R intracellular), R-EN ICmyr, R-EN ICmyr(YF) (Y950F; IRS binding deficient), R-EN ICmyr(KA) (K1003A; kinase dead) and R-EN IC (non-myristoylated intracellular IGF1R) cells were grown under anchorage-independent conditions for 24 h in DMEM containing 0.25% serum. Cells were lysed and subjected to western blotting. **(d)** The same eight cell lines were plated in soft agar and assessed for colony formation ($n = 3$; *represents $P < 0.0001$ by the Student's t -test).

bottom panel). Reciprocal immunoprecipitation using α -IRS1 antibodies confirmed that EN could be co-precipitated in association with IRS1 (Figure 2b). These results indicate that tyrosine phosphorylated IRS1 associates with activated EN in high molecular weight cellular complexes.

An EN/IRS1/IGF1R tripartite complex is essential for transformation

We previously reported that a fraction of EN is localized to the plasma membrane along with activated IGF1R (Martin *et al.*, 2006). However, repeated attempts to co-precipitate EN and IGF1R have been unsuccessful, likely due to the transient nature of the interaction as reported for IGF1R and IRS1 (Myers *et al.*, 1993). As an alternative strategy to visualize EN/IGF1R interactions, we used bimolecular fluorescence complementa-

tion, in which putative interactors are individually fused to separate halves of fluorescent Venus protein and co-expressed in live cells. If the two proteins interact, the Venus protein recombines and fluorescence is reconstituted (Kerppola, 2009). EN fused to the N-terminal Venus (EN-V1) was co-expressed with a second copy of EN fused to C-terminal Venus (EN-V2), demonstrating EN self-association primarily in the cytoplasm (Figure 3a, left panel). Co-expression of V1- and V2-linked to the non-dimerizing EN-V80E mutant (Tognon *et al.*, 2004) showed negligible background fluorescence (data not shown). We then co-expressed EN-V1 along with ICmyr fused to V2 (ICmyr-V2), and detected a robust interaction at the plasma membrane (Figure 3a, right panel). This indicates that EN and IGF1R colocalize at the membrane. Since EN interacts with IRS1 in high molecular weight complexes (Figures 2a and b), we wondered whether IRS1 acts as a bridge to

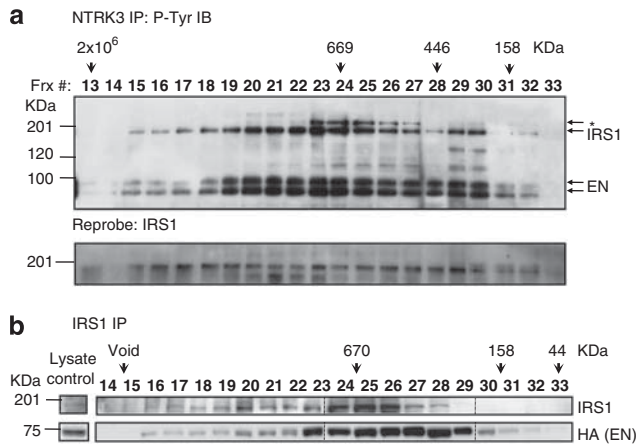


Figure 2 Tyrosine phosphorylated IRS1 interacts with EN in large size FPLC fractions. NIH3T3-EN cells were lysed in a buffer containing 10mM Tris, 150mM NaCl, 1.5mM MgCl₂ and 0.4% NP-40. FPLC was performed with a Superose 6HR10/30 gel filtration column (GE Healthcare, Mississauga, ON, Canada), 0.5ml fractions were collected and anti-NTRK3 immunoprecipitations were performed. Fraction numbers and molecular weights are indicated above the fraction numbers. (a) An anti-phosphotyrosine (P-Tyr; 4G10) antibody was used to probe the membrane. The majority of tyrosine phosphorylated EN elutes in fractions corresponding to the 150 to 1000kDa size range. Two major tyrosine phosphorylated proteins co-immunoprecipitate with EN. *An unidentified 200kDa band. The 180-kDa phosphorylated co-immunoprecipitated protein was identified as IRS1 by western blotting (bottom panel). ETV6-NTRK3 doublet = EN. (b) Reciprocal co-immunoprecipitations were performed on NIH3T3 cells stably expressing HA-tagged EN to confirm the interaction observed in (a). IRS1 conjugated beads (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to immunoprecipitate IRS1. Anti-IRS1 (C-20; Santa Cruz) and anti-HA (Roche, Laval, Canada) antibodies were used to detect IRS1 and the co-immunoprecipitating HA-EN (top and bottom panels, respectively).

localize EN to IGF1R to the membrane to activate PI3K-Akt and mediate transformation. Since, as mentioned, IGF1R could not be readily co-precipitated with EN in FPLC fractions, we instead assessed effects of IGF1R kinase inhibition on EN/IRS1 complex formation. EN-transformed NIH3T3 cells were treated with BMS-536924, a chemical inhibitor that blocks both IGF1R and insulin receptor (INSR) kinase activity with nM potency (Wittman *et al.*, 2005), and protein lysates were subjected to FPLC fractionation. Concentrations of BMS-536924 used for these studies selectively inhibit IGF1R and INSR, but not other tyrosine kinases (Wittman *et al.*, 2005). Although IGF1R remained in high molecular weight fractions under both conditions (Figure 3b, top panels), there was dramatic redistribution of both EN and IRS1 from high to low molecular weight fractions under BMS-536924 treatment (Figure 3b, middle and bottom panels). These smaller sized fractions likely represent dimeric or monomeric forms of EN and IRS1 (Tognon *et al.*, 2004), indicating that IGF1R activation is required to maintain EN and IRS1 in high molecular weight complexes.

One possibility from the above findings is that IRS1 membrane localization alone can overcome the requirement for kinase active IGF1R in EN membrane

localization and transformation. To test this, we generated an HA-tagged IRS1 construct that is myristoylated to target it to the plasma membrane (myrIRS1). As shown in Figure 3c, expression of myrIRS1 in R- and R+ cells not only increased total IRS1 levels but also its distribution to membrane fractions. However, this did not restore EN transformation activity to R-EN cells, and in fact appears to decrease soft agar colony formation of both R-EN and R+ EN cells (Figure 3d). Again, these data point to an essential role for a functional IGF1R axis in EN transformation. To confirm this, we assessed effects of BMS-536924 on EN-mediated survival in anchorage-independent (non-adherent) cultures. While NIH3T3-EN cells showed increased survival in non-adherent cultures compared with vector control cells (Supplementary Figure S4a), BMS-536924 reversed non-adherent survival of EN cells at 24h as assessed by poly (ADP-ribose) polymerase (PARP) and caspase-3 cleavage (Supplementary Figure S4b). Moreover, BMS-536924 dramatically reduced soft agar colony formation of EN-transformed cells (Supplementary Figure S4c). This is supported by our recent report that BMS-536924 blocks EN transformation of breast epithelial cells *in vitro*, and that the related compound, BMS-754807, significantly reduces EN-driven tumor growth *in vivo* (Tognon *et al.*, 2011). Taken together, these results strongly indicate that IGF1R kinase activity is essential for EN transformation, which is consistent with a model whereby EN is maintained in plasma membrane complexes in association with IRS1 and IGF1R.

EN interacts with IRS4 in HEK293 cells and this is reversed by BMS-536924

To independently evaluate EN/IRS/IGF1R complexes, we used HEK293 cells transiently transfected with HA-tagged EN in the presence and absence of BMS-536924. Tandem mass spectrometry analysis was used to compare proteins bound to HA-EN under both conditions. In untreated cells, we observed EN in high abundance as well as the previously described EN interactor, PLC γ (Wai *et al.*, 2000) (data not shown). Strikingly, the most abundant EN interactor in untreated cells was another IRS family member, IRS4. Among IRS family members, HEK293 cells express predominantly IRS4 as well as low levels of IRS2 (Figure 4a). Like IRS1 (and IRS2), IRS4 contains a PTB domain, which is required for IRS1 binding to the C-terminal NPXY site of EN (Lannon *et al.*, 2004; Lannon and Sorensen, 2005). Two independent experiments identified IRS4 in HA immunoprecipitates from EN-expressing cells but not from control cells (coverage 35 and 23%, and Mascot scores 991 and 392, respectively). Under BMS-536924 treatment the averaged IRS4 peptide areas (five peptides) decreased significantly upon BMS-536924 treatment to ~0.2 of the untreated samples (Figure 4b), suggesting that IGF1R kinase inhibition rapidly destabilizes the EN/IRS4 interaction. Representative ion chromatograms for one of the IRS4 peptides are shown in Supplementary Figure S5. These findings were validated by immunoprecipitation, whereby BMS-

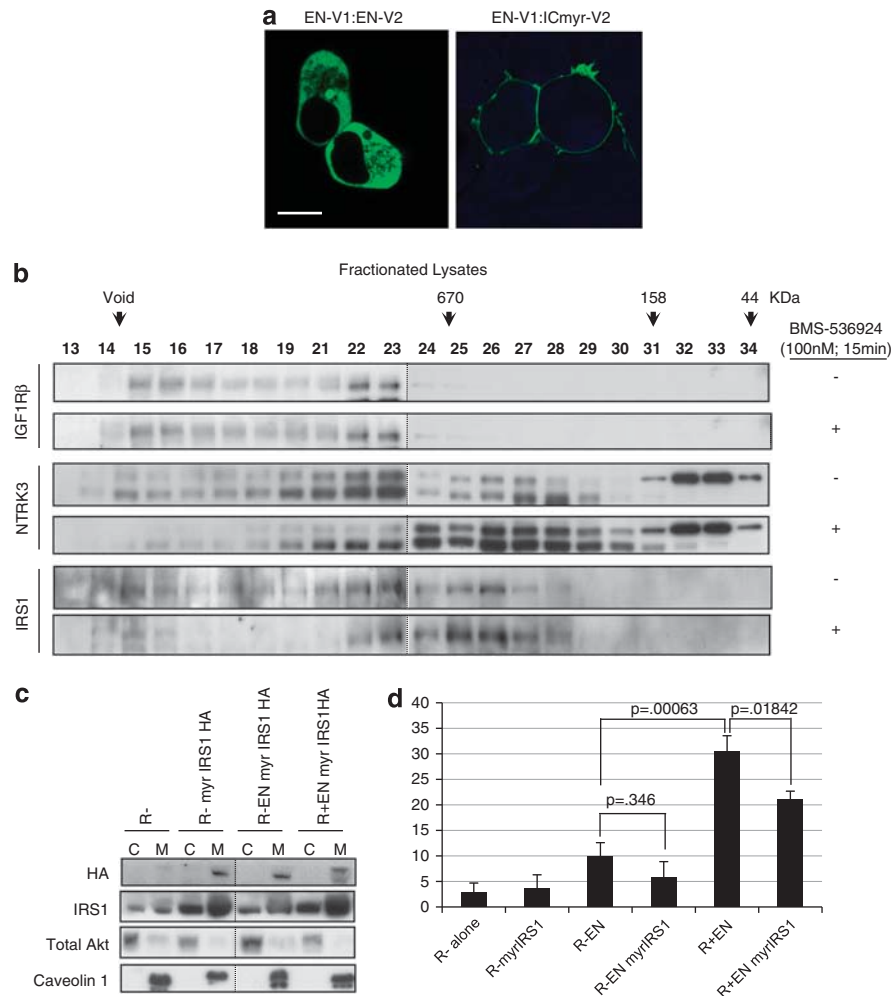


Figure 3 (a) Results of bimolecular fluorescence complementation assays showing colocalization of Venus1-tagged EN (EN-V1) with Venus2-tagged EN (EN-V2) and EN-V1 with Venus2-tagged myristoylated IGF1R intracellular domain (ICmyr-V2) in transiently transfected HEK293 cells. Interaction of EN-V1 with EN-V2 was observed to localize to the cytoplasm (left) whereas EN-V1 interaction with ICmyr-V2 was localized to the plasma membrane (right). (b) Inhibition of IGF1R by BMS-536924 shifts EN protein from larger to smaller sized complexes. Western blot analysis was performed on FPLC fractions collected from EN-expressing cells in the absence (–) and presence (+) of the IGF1R/IR kinase inhibitor BMS-536924 (100 nM; 15 min treatment). Cells were lysed and fractionated as described in Figure 2. IGF1Rβ protein was observed in mid-to-high molecular weight fractions under both conditions. Under non-treated conditions, EN and IRS1 proteins were observed in mid-high sized fractions. Upon BMS-536924 treatment, EN and IRS1 proteins shifted from mid-high to mid-low sized fractions suggesting IGF1R kinase inhibition had dramatic effects upon EN-IRS1 complex formation. (c) Expression of myrIRS1 does not enable EN to transform IGF1R null cells. A myristoylated-IRS1 construct was made and expressed in R–, R–EN and R+EN cells. Cytoplasmic and membrane fractions were isolated from cells using the protocol described in Martin *et al.* (2006). IRS1 was found at a high level in the membranes of myrIRS1-expressing cells. Total Akt and caveolin antibodies were used to identify the cytoplasmic and membrane fractions, respectively. (d) The myrIRS1-expressing cells along with their control counterpart were plated in soft agar and assessed for colony formation ($n = 3$). No significant difference was observed in myrIRS1-expressing R–EN cells compared with R–EN alone ($P = 0.346$). As previously reported (Morrison *et al.*, 2002), statistical differences were observed between R–EN and R+EN cell lines ($P = 0.00063$), but not between R+EN and R+EN myrIRS1 cells ($P = 0.01842$; two-tailed Student's *t*-test).

536924 markedly decreased IRS4 and IRS2 levels co-precipitating with EN (Figure 4c). Finally, we confirmed that in EN-transformed NIH3T3 cells, BMS-536924 markedly reduces co-precipitation of IRS1 with EN (Figure 4d), indicating that IGF1R activity is necessary to maintain EN/IRS1 interactions. These findings underscore the importance of interactions between EN, IRS1 and IGF1R for EN-mediated transformation, and demonstrate how disrupting these interactions blocks EN transformation.

Conclusions

The data presented here support a model whereby the formation of a tripartite complex containing IGF1R, IRS1 (or other IRS proteins) and EN is critical for EN-mediated membrane localization and transformation. Several effects of the BMS-536924 inhibitor on complex formation provide clues to the underlying mechanism. First, BMS-536924 treatment resulted in a dramatic and rapid shift of EN and IRS1 from large to

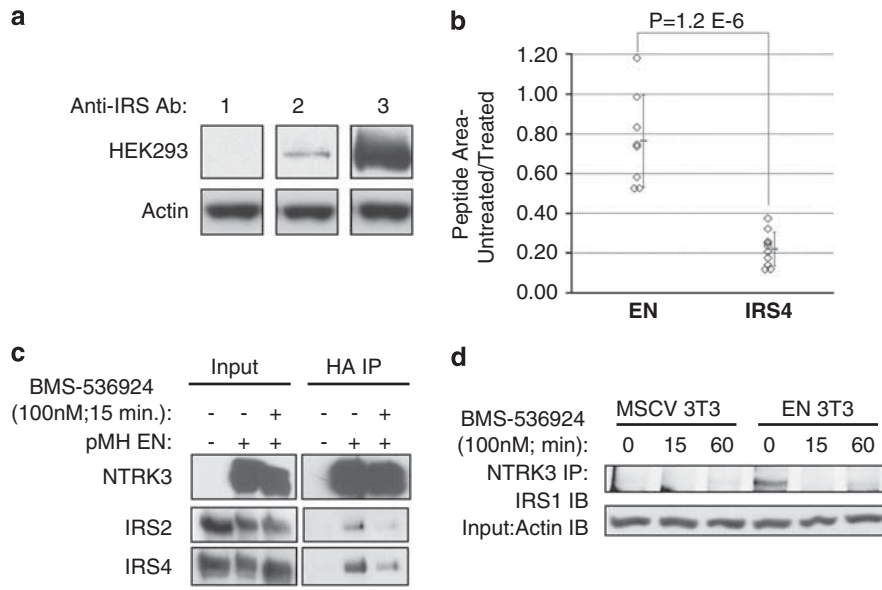


Figure 4 Inhibition of IGF1R by BMS-536924 disrupts IRS-EN interactions. **(a)** IRS isoform expression in HEK293 cells. IRS4 is highly expressed in HEK293 cells. **(b)** Untreated/treated ratios of peptide peak area values for EN and IRS4 peptides identified through mass spectrometry analysis (see also Supplementary Table S1). Treatment = 100 nM BMS-536924 (15 min). A two-tailed Student's *t*-test was performed to determine significance ($P=1.2E-6$). **(c)** BMS-536924 treatment decreases the amount of IRS2 and IRS4 co-immunoprecipitating with HA-tagged EN transiently expressed in HEK293 cells. **(d)** BMS-536924 treatment inhibits the co-immunoprecipitation of IRS1 with EN in NIH3T3 cells. An anti-NTRK3 antibody (C-15; Santa Cruz Biotechnology) was used for immunoprecipitations performed on control (MSCV) or EN-expressing NIH3T3 cells that were either untreated (0) or treated for 15 or 60 min with 100 nM BMS-536924. IRS1 (C-20; Santa Cruz Biotechnology) antibodies were used to detect IRS1 in complex with EN. BMS-536924 treatment blocked IRS1's ability to interact with EN. Lysates were probed for actin to ensure equal protein input for the immunoprecipitations.

smaller FPLC fractions, while IGF1R remained in larger sized fractions. Second, BMS-536924 promoted the release of IRS adaptor proteins from EN, as assessed by mass spectrometry and other studies. Therefore, one possibility is that IGF1R-mediated post-translational modifications of IRS1 are necessary for EN/IRS1 interactions to occur. Post-translational modifications may stabilize EN/IRS1 complexes at the membrane to optimize linkage to critical pathways such as PI3K-Akt. We previously reported that EN/IRS1 complexes are maintained in R-EN cells (Morrison *et al.*, 2002), suggesting IGF1R-independent formation of these complexes. However, BMS-536924 blocks both IGF1R and INSR; and therefore, an additional possibility is that INSR can also modify IRS1 to drive complex formation, but does not support overall EN transformation. Therefore, a second possibility is that IRS proteins deliver EN to the plasma membrane to associate with activated IGF1R, and that EN/IGF1R complexes (and not EN/INSR complexes) are critical for transformation. We are currently exploring these possibilities. The vast majority of known dominantly acting oncoproteins, including activated Ras, c-Src, SV40 large T antigen, and EWS-FLI1, PAX3-FKHR and EN fusion proteins fail to transform mouse embryo fibroblasts lacking IGF1R (Baserga, 2005). Our work provides a plausible mechanism to explain the phenomenon of IGF1R dependency in EN transformation, and suggests that IGF1R kinase inhibitors, through their

ability to rapidly disrupt EN/IRS complex localization to the plasma membrane, could possess significant clinical efficacy in EN-driven tumors. Moreover, the data reported here suggest that the presence of the EN oncoprotein may identify a subset of tumors with enhanced sensitivity to inhibitors of the IGF1R receptor family. It will be important to determine if this mechanism extends to other IGF1R-dependent oncoproteins, particularly membrane-associated chimeric tyrosine kinases.

Conflict of interest

The authors declare no conflict of interest.

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