Characterisation of the cell membrane associated products of the Neuregulin 4 gene.

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Running Title: Characterisation of membrane associated NRG4 variants.

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Abstract

The *NRG4* gene is a member of a family of four genes which encode a class of epidermal growth factors. This gene has been reported to express a protein designated here as NRG4A1. We describe here a novel splice variant of the NRG4 gene, NRG4A2 which encodes a C-terminal region containing a predicted type I PDZ binding peptide. Both NRG4A1 and NRG4A2 were shown to be expressed on the cell surface, as expected by the presence of a predicted transmembrane sequence, and were modified at a single *N*-linked glycosylation site in the extracellular domain. Significant stabilisation of expression of both proteins was seen in the presence of the proteosome inhibitor MG-132 suggesting that they are normally degraded by this system. N-terminal cleavage was inhibited in both isotypes by the broad-spectrum matrix metalloproteinase inhibitor, galardin (GM 6001). A glycosylated, secreted form of NRG4A1 was detected in the cell medium which showed biologically activity in two assays, phosphorylation of the HER4 receptor and stimulation of neurite formation in PC-12 cells stably expressing HER4. Transfection and expression of GFP-tagged proteins and immunofluorescent staining with specific anti-peptide antibodies showed that NRG4A1 is localised to membrane ruffles while NRG4A2 has a more punctuate membrane distribution.

The neuregulins (NRGs) are a family of growth factors which possess a wide range of possible activities including promoting cell growth, differentiation, migration, apoptosis and adhesion (Falls, 2003). There are four mammalian NRG genes (*NRG1-4*) each of which can encode a homologous EGF-like domain which is the minimum requirement for the stimulation of ErbB receptor tyrosine kinases. NRGs 1 and 2 interact with ErbB3 and ErbB4 while NRG3 and NRG4 are reported to bind only ErbB4 (Hobbs et al., 2002). Each NRG gene has a characteristic pattern of expression in normal tissues, *NRG1-3* are all expressed in the nervous system while *NRG4* was detected in a limited number of adult tissues but not the brain (Harari et al, 1999). To date, only one product has been reported for the *NRG4* gene which encodes a 115 amino acid transmembrane protein containing an EGF domain homologous to the other NRGs but outside this region shares little sequence homology with the other NRGs (Buonanno and Fishbach, 2001). It is probable that NRG4 is synthesised as a pro-form, similar to other ErbB ligands, with which it shares structural similarity. Hydropathy plot analysis predicts a single transmembrane region between amino acids 64-83 and sequence analysis predicts an EGF domain at the immediate N-terminus of the protein (Harari et al. 1999) which contains a single N-linked glycosylation site (NYT) located at residue 39 between the fifth and sixth cysteine, a feature found at the N-terminal of the EGF domain of NRG2 (Higashiyama et al, 1997) but not in any of the other EGF receptor ligands. This is followed by a relatively short serine rich region representing a putative proteolytic site. Inspection of the sequence of NRG4 shows that this peptide lacks both a N-terminal hydrophobic signal sequence and a run of apolar residues which are required for correct insertion into the membrane. It is unclear therefore how NRG4 is inserted into the membrane and how the ectodomain is shed (as is the case for some NRG1 isoforms, Wakatsuki

et al, 2004) to produce an ErbB 4 receptor activating ligand. Harari et al. 1999 who reported the first sequence of rat NRG4 showed that a refolded peptide representing the EGF domain of mouse NRG4 stimulated the phosphorylation of HER4 and the downstream second messenger protein Mitogen Activated Protein Kinase (MAPK).

Extensive alternative mRNA splicing and the use of at least six sites of initiation of transcription in NRG1 allows the production of more than 15 variants (Harrison and Law, 2006). NRG2 is also alternatively spliced to produce α and β variants (Yamada et al. 2000) and recently isoforms of NRG 3 have been described (Carteron et al. 2006). There have previously been no reports of NRG4 splice variants. In previous work on NRG4 expression in breast cancer we observed using RT PCR additional NRG4 mRNA products (Dunn et al., 2004). Here we characterise two of these variants which both posses transmembrane sequences; the previously described NRG4 variant described by Harari et al, (1999) which we designate NRG4A1 and a novel 270bp transcript designated NRG4A2.

RT PCR on the breast cancer cell line ZR 75 using full length NRG4A1 primers revealed two NRG4 species one of 345bp (NRG4A1 accession number BC017568) and the other a 270pb (NRG4A2 accession number AM392364) (Figure 1A). When sequenced the second product showed 100% identity to NRG4A1 from amino acids 1 to 85 and then six unique C-terminal amino acids (CGNTCM) which are predicted to be intracellular (Figure 1 B and C). The final four amino acids (NTCM) are predicted to encode a type I PDZ binding peptide (see Harris and Lim 2001 for criteria). Transient transfection of full length C-terminally tagged GFP fusions of NRG4A1 and A2 transcripts into Cos-7 cells and immunoblotting with an anti-GFP antibody

revealed three molecular weight species for each NRG4 isoform only in the presence of the proteosome inhibitor MG-132 (Figure 2A). It has also been reported that the expression of a splice variant of NRG3 (hFBNRG3) which is found in human foetal brain is enhanced in the presence of proteosome inhibitors and the authors suggest that proteosomal degradation of this protein may be indirectly mediated by ubiquitination (Carteron et al., 2006). Both NRG4 isoforms contains lysine residues (NRG4A1/A2 K14, K57 and additionally NRG4A1 K83) which might be targets for ubiquitination (although only K83 is predicted to be intracellular). The high molecular weight smears seen in figure 2B may represent ubiquitinated NRG4A proteins but this will require further investigation. For each splice variant, a band of the correct predicted size of the recombinant protein (NRG4A1, 39.5kDa and NRG4A2, 36.9kDa) was observed as well as a fainter doublet of higher molecular weight. The anti-127 antibody, raised using a synthetic peptide from the common N-terminal sequence of the isoforms, (Figure 1C) detected the lower band for each isotype reproducibly but the higher molecular weight doublet inconsistently between different experiments (Figure 2B) which may indicate variable proteolytic or other post-translational modification occurs. The NRGA1 specific antibody, anti-128, only detected NRG4A1 (Figure 2C).

The amino acid sequence of the NRG4 isoforms indicate that a single potential N-linked glycosylation site is present (NYT, Figure 1C), in order to establish whether any of the observed bands represented glycosylated species the proteins were treated with PNGase F and a shift of the higher band in the doublet to the lower band in the doublet confirmed N-linked glycosylation had occurred in both the NRG4 isoforms (Figure 2D).

We next determined whether the variants were expressed on the cell membrane and whether the N-terminus was orientated on the extracellular surface. Sequence analysis using the criteria of von Heijne predicted that these would be a type I transmembrane proteins with the N-terminus exposed on the extracellular space (von Heijne 2006). Cos-7 cells were transfected with the GFP-tagged fusion, fixed and either permeabilised with detergent or left unpermeabilised and then stained with the anti-127 antibody. Cell surface expression was seen in the GFP tagged images but the anti-127 antibody only detected the protein in permeabilised but not unpermeabilised cells. We hypothesised that this might be due to rapid shedding of the extracellular domain. The matrix metalloproteases ADAM 17 and ADAM 19 have been implicated in the shedding of Neuregulins in cells in culture (Shirakabe et al., 2001; Montero et al., 2000). We therefore treated the cells with galardin, a broad spectrum metalloprotease inhibitor which inhibits TACE/ADAM type proteases. In this case, the N-terminal antibody now gave a strong signal in unpermeabilised cells indicating that galardin allowed the accumulation of uncleaved protein and that the N-terminus was exposed on the cell surface (Figure 3A).

The two variants differed only in their intracellular domains and, as NRG4A2 possessed a PDZ binding domain, we assessed whether they might have different plasma cell membrane localisations. Transient transfection of Cos-7 cells with constructs of NRG4A1 and NRG4A2 lacking the GFP tag (Figure 3A) were carried out in the presence of galardin and the proteins detected using the anti-127 antibody on unpermeabilised cells. At 24 hr post transfection NRGA1 was localised to membrane ruffles while NRG4A2 had a fine uniform punctuate appearance (Figure 3B).

The data presented above (Figure 2 and 3) suggested that a glycosylated, extracellular fragment containing an EGF domain would be present in the cell growth medium. In order to test this, conditioned media from Cos-7 cells transiently transfected with NRG4A1-GFP was collected, concentrated, loaded onto a Concanavalin A (Con A) column and bound glycoproteins were eluted. This eluate was added to NIH3T3 HER4 cells and Western blotting with anti-phosphotyrosine antibodies revealed a strong band at the predicted molecular weight of the ErbB4 receptor not present in the mock treated cell lysate (Figure 4A lanes 1-3). As a further control, refolded and oxidised NRG4 peptide (as described by Harari et al. 1999) also stimulated phosphorylation of a similar species (Figure 4A lane 4).

It has been demonstrated that NRG1 can elicit neuronal outgrowth in PC-12 HER4 cells (a cell line derived from a rat adrenal medullary pheochromocytoma cells and stably transfected with HER4, Vaskovsky et al., 2000). On addition of the Con A eluate (from NRG4A1-GFP transfected cells) to PC-12 HER4 cells, neurite outgrowth was observed by 72hr but not on the addition of the eluate from untransfected cells (Figure 4B). Concentrated conditioned medium from NRG4A1-GFP transfected cells which had been treated with galardin failed to stimulate neurite outgrowth (Figure 4B) indicating that shedding was a result of proteolytic cleavage. Chemically synthesised and refolded NRG4A1 was used as a positive control for neurite outgrowth.

In summary these data reveal that in spite of lacking a hydrophobic N-terminal hydrophobic signal sequence NRG4A1 and A2 are transported to the cell surface as glycosylated peptides and that shedding of a biologically active ligand occurred as a consequence of proteolytic cleavage probably by a TACE/ADAM type protease. We show here that an EGF-like ligand is glycosylated at an asparagine residue within the

EGF domain. Inspection of the three dimensional structure of TGF alpha bound to the EGF receptor (Garrett et al. 2002) shows that the equivalent residue to the asparagine in the glycosylation site of NRG4 is exposed on the surface and not buried in the interaction site (see Supplementary Figure 1) suggesting that this modification should not sterically hinder binding. Indeed it is clear from the results presented above that both glycosylated (Con A purified) and unglycosylated (chemically synthesised) NRG4 are both active. A potential N-linked glycosylation site is also found in NRG2 immediately following the first cysteine residue in the EGF domain (Higashiyama et. 1997) but it is not known if this is glycosylated. Molecular modelling also demonstrates that if this were modified it would be unlikely to prevent receptor binding. Finally, there are indications that the two isoforms have subtly different patterns of expression on the cell surface as a consequence of the differential splicing of their C-terminal sequences.

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Supplementary information is available on http://www.nature.com/onc/index.html.

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Titles and legends to figures

Figure 1.

Identification and sequence alignment of NRG4A2 from ZR 75 breast cancer cell line. ZR 75 cells were cultured to 80% confluency in DMEM, 10% FCS (Gibco), 2mM L-glutamine, 50ug/ml penicillin and 50ug/ml streptomycin. RNA (RNeasy Mini Kit and QIAshredder, Qiagen) was reverse transcribed and RT-PCR was carried out using A) NRG4A1 (lane 1) and NRG4A2 (lane 2) specific primers. Forward primer for both isotypes 5' NRG4 F 5'C ACCATGCCAACA GATCACGAAGAGCC 3'; the reverse primer for NRG4 A1; 5'GTGTTGTTCATGACTGTGGTGG 3'; NRG4 A2 5' CTA CATGCATGTGTTACCACACC 3'. The PCR products were separated using 3% agarose gel electrophoresis, the band(s) excised and cleaned (QIAquick gel extraction kit, Qiagen, UK). B) Sequencing of the PCR products (Lark Technologies, UK) allowed alignment of NRG4A1and NRG4A2.C) Alignment of the translated transcripts showing the position of the peptides used to raise the N-terminal pan anti-NRG4 rabbit antibody (anti 127), the C-terminal NRG4A1 specific rabbit antibody (anti-128).The predicted transmembrane region, the *N*-linked glycosylation site, the predicted PDZ domain and the peptides use for antibody production are underlined. An asterix indicates where the two sequences diverge.

Figure 2.

Identification, deglycosylation and protease inhibition of NRG4A1/A2 transcripts. Both NRG4A1 and NRG4A2 full length PCR products were directionally subcloned into pEGFP-N1 (Clontech) using SacII/KpnI sites in frame with a C-terminal GFP tag. Cos-7 cells were transfected (Fugene 6, Roche) in 2cm dishes with 0.2ug NRG4

A1 GFP (panel A lanes 2-3), NRG4A2 GFP (panel A lanes 5-6) or GFP only (panel A lane 7) for 24hrs and 20μM of the proteosome inhibitor MG-132 (Sigma,U.K) was added (lanes 1,3, 4 and 6,) for 18hr prior to lysis. The cells were lysed by directly adding 2x Laemelli sample buffer and 15% SDS PAGE was carried out on the samples. Immunoblotting onto nitrocellulose (Amersham, UK) and probing with A) 3E1 anti mouse GFP monoclonal antibody B) rabbit anti-127 (NRG4 pan specific antibody) and C) rabbit anti-128 (NRG4A1 specific) was carried out. For deglycosylation, cells were lysed (50mM Tris, 0.15M NaCl, 1% Triton X-100, 5mM EDTA, plus protease inhibitors) and proteins denatured by the addition of 0.5%SDS, 40mM DTT at 100^{o}C for 10 mins. 50mM $NaHPO_4 2H_20$ 10%, pH 7.5 and 10% Nonidet was added to the samples followed by 2000U PNGase F (New England BioLabs,UK) and incubation for 4hr at 37^{o}C prior to 15% SDS PAGE as above and detection by the 3E1 antibody. D) GFP NRG4A1 in the absence (lane1) and presence (lane 2) of PNGase F, GFP NRGA2 in the absence (lane 3) and presence (lane 4) of PNGase F. E) 10μM galardin (and MG-132 as above) was added to Cos-7 cells transfected with either GFP NRG4A1 (lane 2) or GFP NRG4A2 (lane 3) (0.1% serum) for 96 hours prior to 15% SDS PAGE and detection with the 3E1 GFP antibody.

Figure 3

NRG4A1 and NRG4 A2 localise to different cell membrane populations.

A) Both NRG4A1 and NRG4A2 full length PCR products were directionally subcloned into the pEGFPN-1 vector or B) pcDNA 3.1D/V5-His-TOPO vector (Invitrogen) and Cos-7 cells were transfected either with NRG4A1 or NRG4A2 as described above for the times indicated and fixed with 4% parafomaldehyde, pH 7.4.

Indirect immunofluorescence was carried out using the rabbit polyclonal anti-127 antibody and anti-rabbit Alexa Fluor 546. A) Cos-7 cells were permeabilised with 0.5% Triton X-100 for 5 min at 0^{o}C or left unpermeablised with or without 10μM galardin in 0.1% serum.B) At 24hr post transfection NRG4A1 is localised to membrane ruffles while NRG4A2 has a more punctuate appearance on the cell membrane.

Figure 4

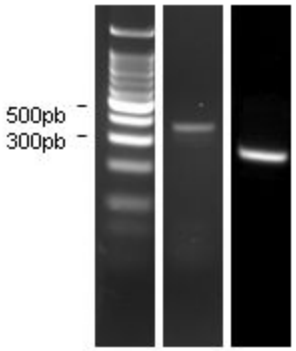
The glycosylated ectodomain of NRG4 A1 promotes neurite extension in PC-12 HER 4 cells and tyrosine phosphorylation of HER 4.

Conditioned media (containing 0.05% FCS) was collected 48hr after transfection of Cos-7 with NRG4A1 GFP, concentrated using a Vivaspin column (MW cut off 3000kDa), and $MnCl_2$ to give a final concentration of 5mM. Con A beads were equilibriated into 0.5M NaCl, 20mM Tris, pH 7.4, 5mM $MnCl_2$, 5mM$CaCl_2$ and incubated with the concentrated conditioned media at 4^{o}C for 2hrs. The beads were washed into the equlibriation buffer (see above) and incubated for 5 mins at 4^{o}C in 1.0M methyl α D mannopyranoside glycoproteins. 100μl of eluted glycoproteins was recovered from the supernatant and 20μl was added A) for 5 mins at 37^{o}C to NIH 3T3 HER 4 cells which were cultured in the same media as Cos–7 cells (see above). The cells were lysed as above and 15% SDS PAGE was carried out. The blotted samples were probed with the anti phosphotyrosine antibody, PY20. B) for 72hr at 37^{o}C to PC-12 HER 4 cells which were cultured to 50% confluency in RPMI, 5% horse serum 10% FCS (Gibco), 2mM L-glutamine, 50ug/ml penicillin and 50ug/ml streptomycin in coverslip dishes as above. 24hrs prior to addition of the conditioned media the culture medium for both cell lines was changed to 0.1% serum. B) Panels

top to bottom, first row - conditioned media (CM) from transfected cells, with Galardin, and with chemically synthesised and refolded NRG4A1 peptide was added to the cells. Panels top to bottom – second row matched controls.

Figure 1

a



b

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actga

c

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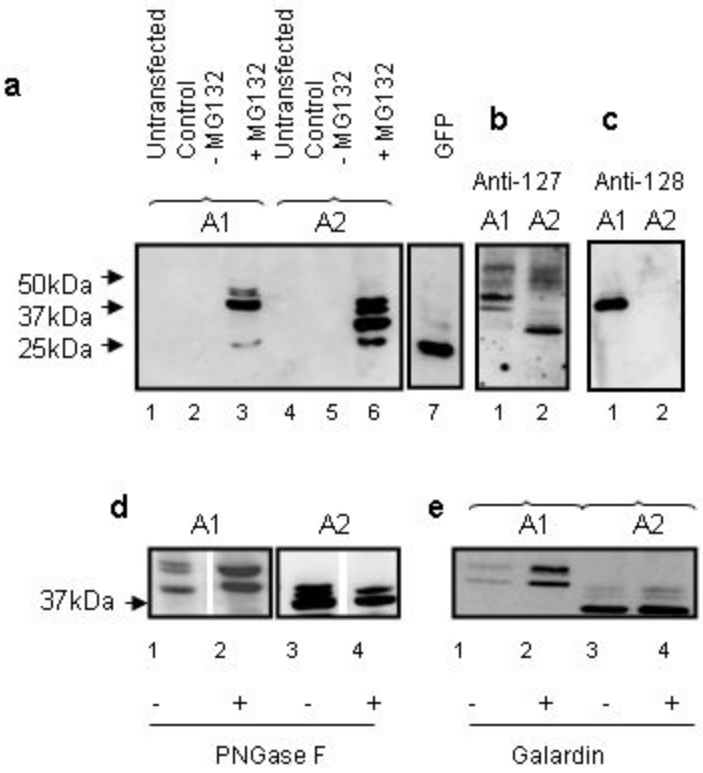
MPTDHEEPCGPSHKSFCLNGGLCYVIPTIPSPFCRCVENYTGARCEEVFLPGSSIQTKSNLFEAFVALAVLVTLIIGAFYFLCRCGNTCM

anti - 127 N-Gly Transmembrane PDZ motif anti - 128

Figure 2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Control
- MG132
+ MG132
Untransfected
Control
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+ MG132
GFP
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Anti-127
c
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A2
A1 A2
A1 A2
50kDa
37kDa
25kDa
1 2 3 4 5 6 7
1 2
1 2
d
A1
A2
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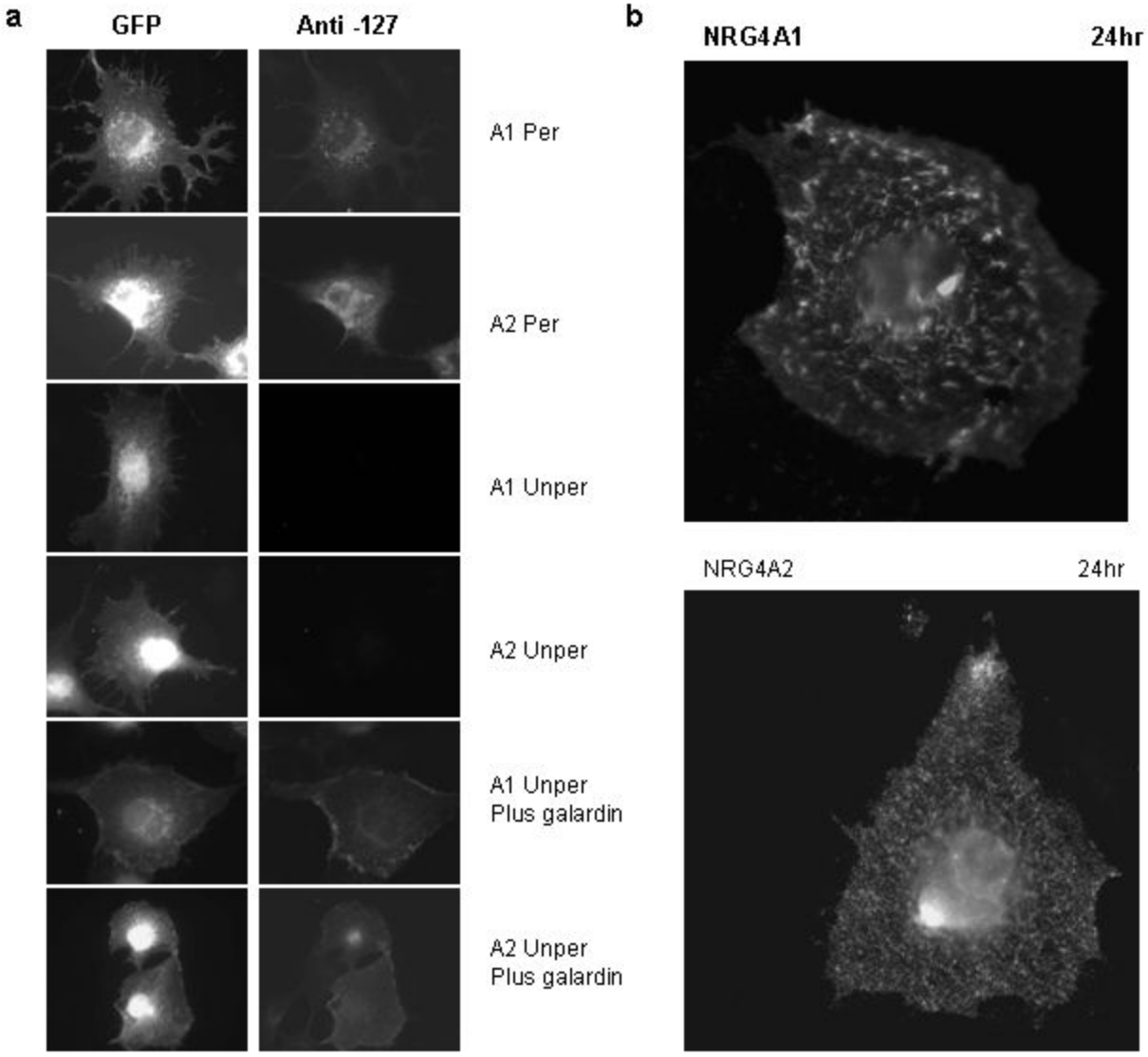
- + - +
Galardin

Figure 3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A1 Unper
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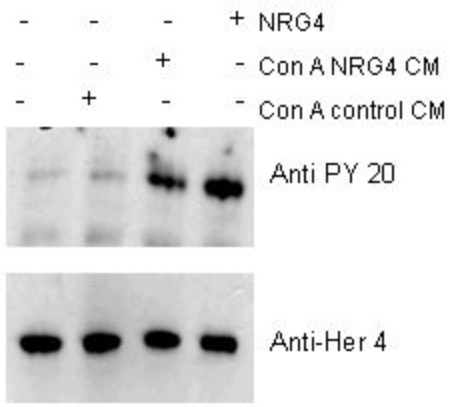
A2 Unper
Plus galardin
b
NRG4A1
24hr
NRG4A2
24hr

Figure 4

a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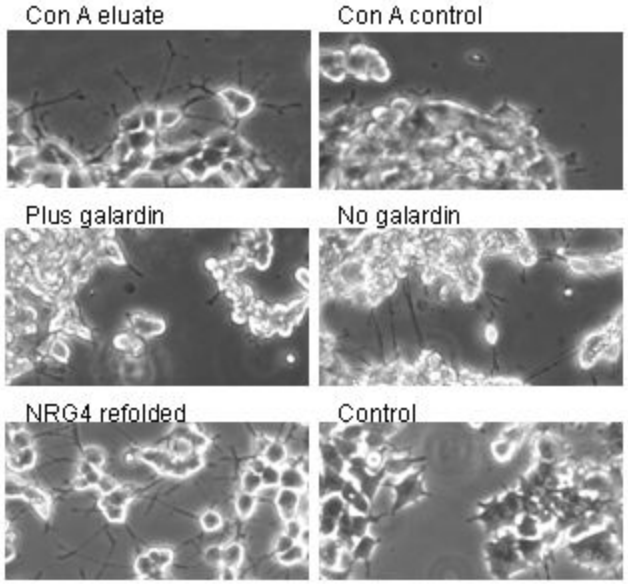
Anti PY 20
Anti-Her 4

b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Con A control
Plus galardin
No galardin
NRG4 refolded
Control