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Title: RGD-based cell ligands for cell-targeted drug delivery act as potent trophic factors

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Keywords: Protein nanoparticles; integrins; RGD; neurons; nanomedicine; biomaterials

Corresponding Author: Prof Antonio Villaverde, PhD

Corresponding Author's Institution: UAB

First Author: Joan Domingo-Espín

Order of Authors: Joan Domingo-Espín ; Valérie Petegnief ; Núria de Vera ; Oscar Conchillo ; Paolo Saccardo; Ugutz Unzueta ; Esther Vazquez ; Juan Cedano ; Luciana Negro ; Xavier Daura ; Hugo Peluffo ; Anna M. Planas ; Antonio Villaverde, PhD; Neus Ferrer-Miralles

Abstract: Integrin-binding, arg-gly-asp (RGD)-containing peptides are the most widespread used agents to deliver drugs, nanoparticles and imaging agents. Although in nature, several protein-mediated signal transduction events depend on RGD motifs, the potential of RGD-empowered materials in triggering undesired cell signalling cascades has been neglected. Using an RGD-functionalized protein nanoparticle we show here that the RGD motif acts as a powerful trophic factor, supporting extracellular signal-regulated kinase 1/2 (ERK1/2)-linked cell proliferation and partial differentiation of PC12 cells, a neuron-like model.

***Cover Letter**

Ms. Ref. No.: JN2012101
Title: RGD-based cell ligands for cell-targeted drug delivery act as potent trophic factors
Nanomedicine: Nanotechnology, Biology, and Medicine

Marianna Foldvari, PhD
Associate Editor
Nanomedicine: Nanotechnology, Biology, and Medicine
http://www.nanomedjournal.com/

Dear Dr. Foldvari,

Thank you very much for your e-mail dated on May 15 and for all the comments on our Ms JN2012101. I believe that by following the given suggestions, the presentation of our data and the whole MS has largely improved, and I hope you will now find it suitable for publication in Nanomedicine: Nanotechnology, Biology, and Medicine. Please find separately our point-by-point responses to all the raised issues and a description of how the formed MS file has been modified accordingly.

Best regards

A. Villaverde

***Detailed Response to Reviewers**

Ms. Ref. No.: JN2012101
Title: RGD-based cell ligands for cell-targeted drug delivery act as potent trophic factors
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Marianna Foldvari, PhD
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Best regards

A. Villaverde

Reviewer #1: It is of interest that HNRK was found to stimulate PC12 cell growth at levels comparable or even higher than those promoted by the nerve growth factor (NGF). The study provides a novel and potentially powerful idea for nanomedicine and namely that the vehicle itself could provide trophic factor support through supporting ERK-linked cell proliferation and partial differentiation. The question remains is what is observed in a cell line reflective of what may occur with primary cells. Stimulation of cell proliferation in tumor cells can occur through a broad range of factors that include growth factors, cell signaling events, cytokines, chemokines and others. If a case is made for these findings to affect neuronal cells than the findings need to reproduced in primary neurons. This is ever important as NGF is well known to promote neuronal differentiation of PC12 pheochromocytoma cells. This has been reported to occur within 5 min after NGF addition. Thus, stating that as yet undisclosed factor engaging these cells is more powerful in its effects than NGF requires cross validation of the data set.
We agree with the referee in that cross validation of data would make our message stronger. We also believe that testing RDG with primary neuronal cultures would probably not the best experimental model as they are highly differentiated cells. We have chosen to offer data showing RDG-promoted ERK activation in primary glial cell cultures (panel C in the new Figure 4). Being suitable for the requested cross validation, these positive results also indicate that the reported trophic effects are not restricted to a specific lineage, in the context of the point 2 raised by the referee #2.

Reviewer #2: The paper is interesting and well written. I have only a few comments.
We appreciate the positive opinion of the referee.

1. Although very well researched, I don't know that it's fair to say that RGD peptides are 'the most universal tags for targeted delivery of drugs and nanoparticles'.
We fully agree with the referee. The sentence in the graphical abstract has been modified as follows: … that RGD peptides, among the most used tags for targeted delivery of drugs and nanoparticles…..

2. I think that other cell types, especially endothelial cells, need to be explored before the RGD cell trophic properties could be defined as of 'strategic interest' in the design of targeted therapeutics.
We agree. We have toned down this sentence as follows: ….potential interest…. However, we have shown that the trophic effect of RGD is not restricted to neuronal lineage, as shown in Figure 4 C by incorporating studies with primary glial cell cultures.

3. Page 3, 2nd paragraph after (Figure 1 c) change 'what' to that and add a semicolon after the parentheses.
Done

Reviewer #3: Specific comments:
1. Page 2, line 3: motive is wrongly given instead of motif. **Done.**
2. Page 3, line 12: motive is wrongly given instead of motif. **Done.**
3. Page 3: The source of the PC12 cell is not mentioned. **Done in the Supplementary Information. We have also indicated the source of the other cell lines employed in the study.**
4. Fig 2.C: In Y axis MTT % must be changed into Viability %. **Done, and also in Figure 4.**
5. Reference 5: Volume and page number must be included. **Done.**
6. Reference 9: Page number must be included. **The book reference has been completed according to the journal style.**
7. More relevant and clear title can be given for Graphical abstract below the image. **Changed into: RGD peptides are potent trophic factors**.

General comments:
1. Most of the abbreviations like ERK (abstract, line No: 6), HNRK, etc. are not explained properly. **ERK was defined in page 2, end of the first paragraph, but we have now defined it again in the abstract to make it clearer. HNRK is not an abbreviation but the name given to the modular protein. We now explicitly indicate the meaning of all the consecutive protein segments. In addition, we have carefully revised the MS to ensure that all the abbreviations are clearly explained.**
2. Figures 3.A, clarity can be improved. **We are not sure if we correctly understand the referee's point. Figure 3A is an in situ immunodetection of MAP-2 in cells growing under 20 % serum and it represents a control for the experiment shown in the figure. The field selected for panel A matches that shown in panel B. In its present form, the figure is reasonably clear and it cannot (and should not) be manipulated without losing its value as a control.**

Graphical Abstract: Text

RGD peptides are potent trophic factors.
We describe here that RGD peptides, among the most used tags for targeted delivery of drugs and nanoparticles promote protoneurite formation, p-ERK1/2-dependent cell division in absence of serum and expression of neuronal markers (MAP-2) in model PC12 cells. The trophic potential of RGD peptides is an unexpected event of critical relevance in the functionalization of drugs and their nanocarriers.

Graphical Abstract: Image

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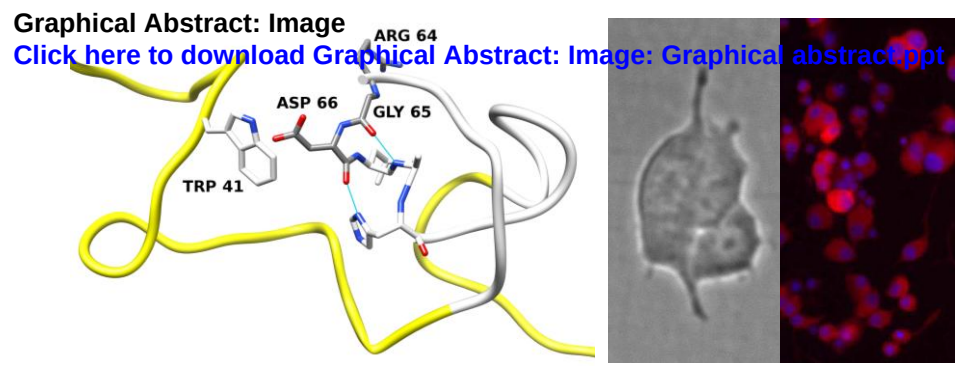

RGD-based cell ligands for cell-targeted drug delivery act as potent trophic factors

Joan Domingo-Espín [1, 2, 3], Valérie Petegnief [4], Núria de Vera [4], Oscar Conchillo-Solé [1], Paolo Saccardo[1, 2, 3], Ugutz Unzueta [1, 2, 3], Esther Vazquez [1, 2, 3], Juan Cedano [5], Luciana Negro [6], Xavier Daura [1,6], Hugo Peluffo [7,8], Anna M. Planas [4], Antonio Villaverde [1, 2, 3 *], Neus Ferrer-Miralles [1, 2, 3]

[1] *Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain*
[2] *Department of Genetics and Microbiology, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain*
[3] *CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Bellaterra, 08193 Barcelona, Spain*
[4] *Departament d'Isquèmia Cerebral i Neurodegeneració, Institut d'Investigacions Biomèdiques de Barcelona (IIBB), Consejo Superior de Investigaciones Científicas (CSIC)-Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain*
[5] *Laboratory of Immunology, Regional Norte, Universidad de la Republica, Gral. Rivera 1350; Salto, 50.000, Uruguay*
[6] *Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain*
[7] *Neurodegeneration Laboratory, Institut Pasteur de Montevideo, CP 11400, Montevideo, Uruguay 4*
[8] *Department of Histology & Embryology, Faculty of Medicine, UDELAR, CP 11800, Montevideo, Uruguay*

[*] **Corresponding author:** A. Villaverde; Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain. Phone:+34 935813086; Fax: +34 935812011. E-mail: antoni.villaverde@uab.cat

Keywords: Protein nanoparticles; integrins; RGD; neurons; nanomedicine; biomaterials

Number of words in the text body, including abstract and figure legends: 1444

Number of Figures: 4

Number of Tables: 0

Number of references: 10

ABSTRACT

Integrin-binding, arg-gly-asp (RGD)-containing peptides are the most widespread used agents to deliver drugs, nanoparticles and imaging agents. Although in nature, several protein-mediated signal transduction events depend on RGD motifs, the potential of RGD-empowered materials in triggering undesired cell signalling cascades has been neglected. Using an RGD-functionalized protein nanoparticle we show here that the RGD motif acts as a powerful trophic factor, supporting extracellular signal-regulated kinase 1/2 (ERK1/2)-linked cell proliferation and partial differentiation of PC12 cells, a neuron-like model.

Innovative medicines aim to the cell-targeted delivery of therapeutic cargos. The concept supporting this approach is that specific binding to selected cell surface receptors should result in improved biodistribution and enhanced cellular penetrability. Since the discovery of the arg-gly-asp (RGD) motif as a potent ligand of cell surface integrins (mainly $\sigma_v ß_3$) (1), RGD-containing peptides have been massively used to control drug biodistribution, being the most employed agents for cell targeting and endosomal delivery (2). As integrins abound in endothelial cells and because of the high vascularisation of tumoral tissues, RGD-mediated drug-delivery is of special interest in cancer therapies (2). Interestingly, some RGD-containing natural proteins, including fibulin-5 (3), osteopontine (4), the angiogenic factor Del1 (5) and the dentin matrix acidic phosphoprotein 1 (DMP1) (6), act as biological effectors through their RGD motifs, at least some of them through the extracellular signal-regulated kinase 1/2 (ERK1/2)-mitogen-activated protein kinase (MAPK) pathway. Therefore, it would not be unexpected that RGD-empowered constructs for drug targeting could trigger side responses in target cells, although this possibility has not been investigated.

HNRK is a short modular protein of 91 amino acids in length, consisting of 4 consecutive functional peptides, namely an endosomolytic poly-histidine peptide (H), a viral nuclear localization signal (N), an RGD-containing site of foot-and-mouth disease virus (FMDV) serotype C1 (R) and a C-terminal poly-lysine (K) peptide (Figure 1 a). In the presence of plasmid DNA, HNRK forms 80 nm-polyplexes efficient in DNA delivery to mammalian cells (7). The transfection efficiency of HNRK nanoparticles is highly dependent on the amount of $\alpha_v \beta_3$ displayed on the cell surface (Figure 1 b), indicating that the RGD stretch determines the capability of the construct to penetrate the target cell and deliver the cargo. Contrarily, HKRN, a modular isoform of HNRK (Figure 1 a) fails to promote high levels of transgene expression (Figure 1 c), a fact that could be attributed to a poor presentation of the cell ligand. In this regard, in a homology model of HNRK, RGD is folded as a mirror-like structure of the parental viral segment, while in HKRN the segment folds in part as a mirror and in part as a fully matching conformation, stabilized by different interactions (Figure 1 d). Interestingly, when exposing PC12 cells to HNRK alone (but not to HKRN), proto-neurite formation was unexpectedly observed (Figure 2 a, and Supplementary figure). In this context, HNRK specifically stimulated PC12 cell growth at levels comparable or even higher than those promoted by the nerve growth factor (NGF) (Figure 2 b, c). Both the morphological modification of PC12 cells and the enhanced cell growth upon exposure to HNRK indicated that this protein could act as a cell trophic factor. Since HKRN, in which the displayed RGD is unable to efficiently bind integrins did not stimulate cell survival,

growth and differentiation, it could be speculated that a functional RGD motif could be responsible for the HNRK-induced trophic effect. This was fully supported by the fact that the HNRK variant HN(RGE)K, in which the RGD sequence was mutated into the non-binding RGE motif, did not show trophic effects (Figure 2 b, c).

To explore the biological effects mediated by HNRK, we analyzed the signaling events in HNRK-exposed PC12 cells through the expression of microtubule associated proteins typically used as neuronal markers. Indeed, HNRK up-regulated microtubule-associated protein-2 (MAP2) (Figure 3) and Tau (not shown). Interestingly, HNRK but not HN(RGE)K also stimulated the phosphorylation of ERK1/2 (Figure 4 a). The degree of ERK1/2 phosphorylation induced by HNRK was lower than that triggered by NGF, while in contrast, the chimeric protein stimulated cell proliferation more than NGF did (Figure 2). These results suggest different effector pathways for NGF and HNRK. PD98059, an inhibitor of the MAPK kinases MEK1 and 2 that phosphorylates ERK1/2, reduced NGF-induced ERK1/2 phosphorylation and fully abolished HNRK-induced ERK1/2 phosphorylation (Figure 4 a). Interestingly, while cell proliferation mediated by HNRK was significantly reduced by more than 50 % by PD98059, this inhibitor only showed a trend for reduction of NGF-induced cell proliferation by around 15 % (Figure 4 b).

Finally, to explore whether the trophic effects of RGD described here could be restricted to a specific cell lineage (neurons) we extended our study to rat primary glial cell cultures enriched in astrocytes. In this additional model, a dose-dependent ERK1/2 phosphorylation was clearly observed upon exposure to HNRK, indicating that the effector role of the peptide motif is instead a generic event (Figure 4c).

The tripeptide RGD is one of the most used cell ligands in Nanomedicine for the targeted delivery of drugs, as associated with chemicals, protein-only constructs, self-assembling peptides, liposomes, dendrimers, carbon nanotubes, magnetic nanoparticles, quantum dots, polymers and other delivery agents (8;9). RGD is also used as a cell binding agent in cell culture and in different approaches of tissue engineering as a functional coating agent to favor cell binding to the substrate and differentiation (10). Therefore, the cell trophic activities of RGD described in the present study are of potential interest when evaluating the biological and therapeutic effects of RGD-based therapies, as the side effects of the vehicle might antagonize or synergistically act with those of the therapeutic cargo. This might be of special interest if the nanoparticle-linked drug is intended to arrest cell growth or is a cell killing agent,

as it usually occurs in antitumoral therapy. Importantly, the catalogues of agents used in nanomedicine for cell targeting are rich in natural or mimetic ligands of receptors for hormones, cytokines or other effectors that might potentially trigger signaling events.

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Legends for the figures

Figure 1: A. Schematic representation of HNRK and HKRN (modified from (7)). B. Relationship between the percentage of integrin positive cells and transgene expression levels mediated by HNRK. C. Percentage of HeLa and GL261 transfected cells, mediated by HNRK-DNA or HKRN-DNA nanoparticles. D. Detail of the residues closer than 4 A to the arg residue in the RGD module (in white, in yellow, the rest of the peptide), modeled using the segment 134-156 from FMDV VP1 (pdb 1QGC). In the insets, superposition of the recombinant RGD modules (blue) over the template (red) used to model it, again residues 134-156 from FMDV VP1 (pdb 1QGC). Details of all the experimental procedures used in the study can be found in the **Supplementary information**.

Figure 2: A. Magnifications of HNRK- and NGF-treated cells (at 100 ng/ml). Scale bar= 20 μm. Proliferation of PC12 cells monitored by cell counting (B) or relative MTT activity (C). Cells were cultured for 7 days in the absence of serum (Cell –S); with 20 % serum (Cell +S); in absence of serum plus NGF; in absence of serum plus either HNRK or HN(RGE)K. Only significantly different pair of comparative data are indicated (p<0.01, n=3).

Figure 3: Immunofluorescence for MAP-2 (red) in cells either maintained under control conditions (20 % serum) (A, B), or treated with NGF (C, D) or HNRK (E, F). Nuclei are stained with DAPI (blue) in B, D, F. Neurite extensions are indicated by arrows. Scale bar= 50 μm.

Figure 4. A. ERK1/2 phosphorylation determined by Western blotting (n=3). β-Tubulin is shown as a gel loading control. B. Cell viability determined by MTT assay. Only significantly different pair of comparative data are indicated (p<0.01, n=3). C. ERK1/2 phosphorylation determined by Western blotting two hours after exposure of astrocyte-enriched glial cell cultures to HNRK (75 ng/μl and 150 ng/μl respectively). β-Tubulin is also shown as a gel loading control. C indicates non-exposed control cells.

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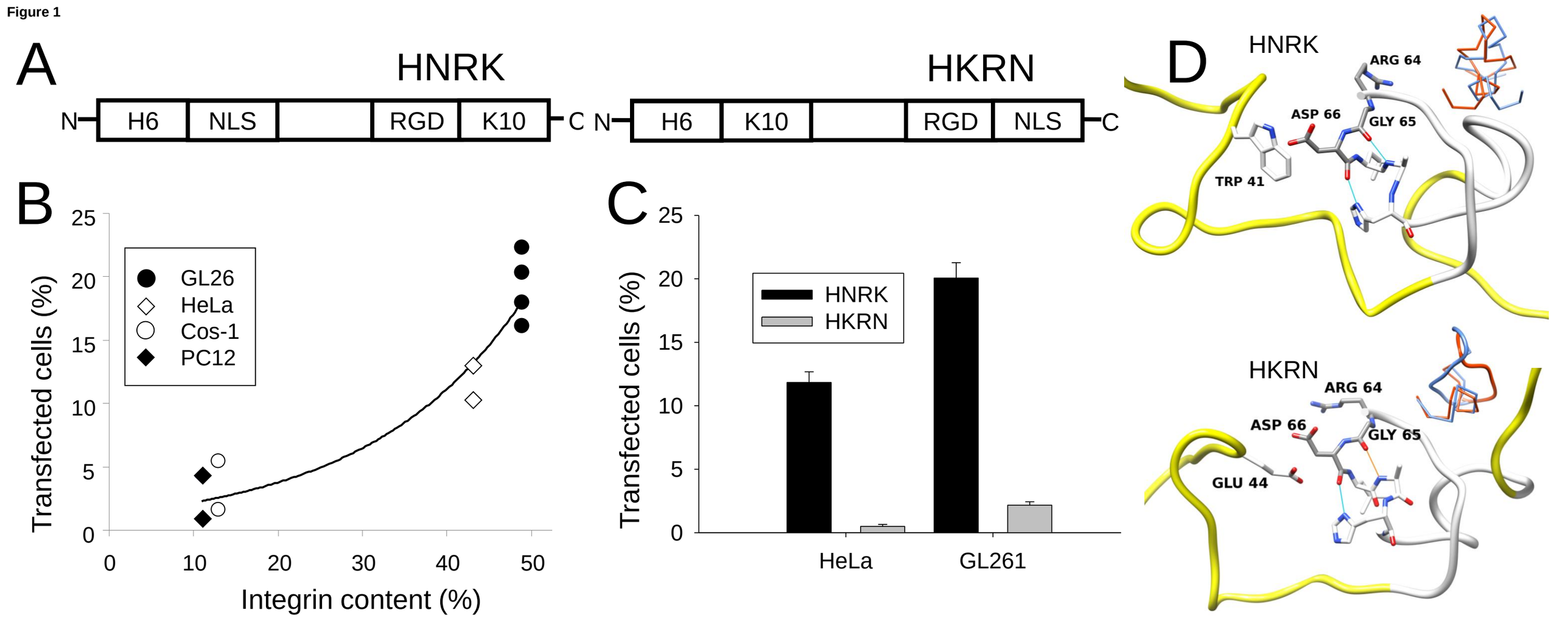
Figure 1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K10
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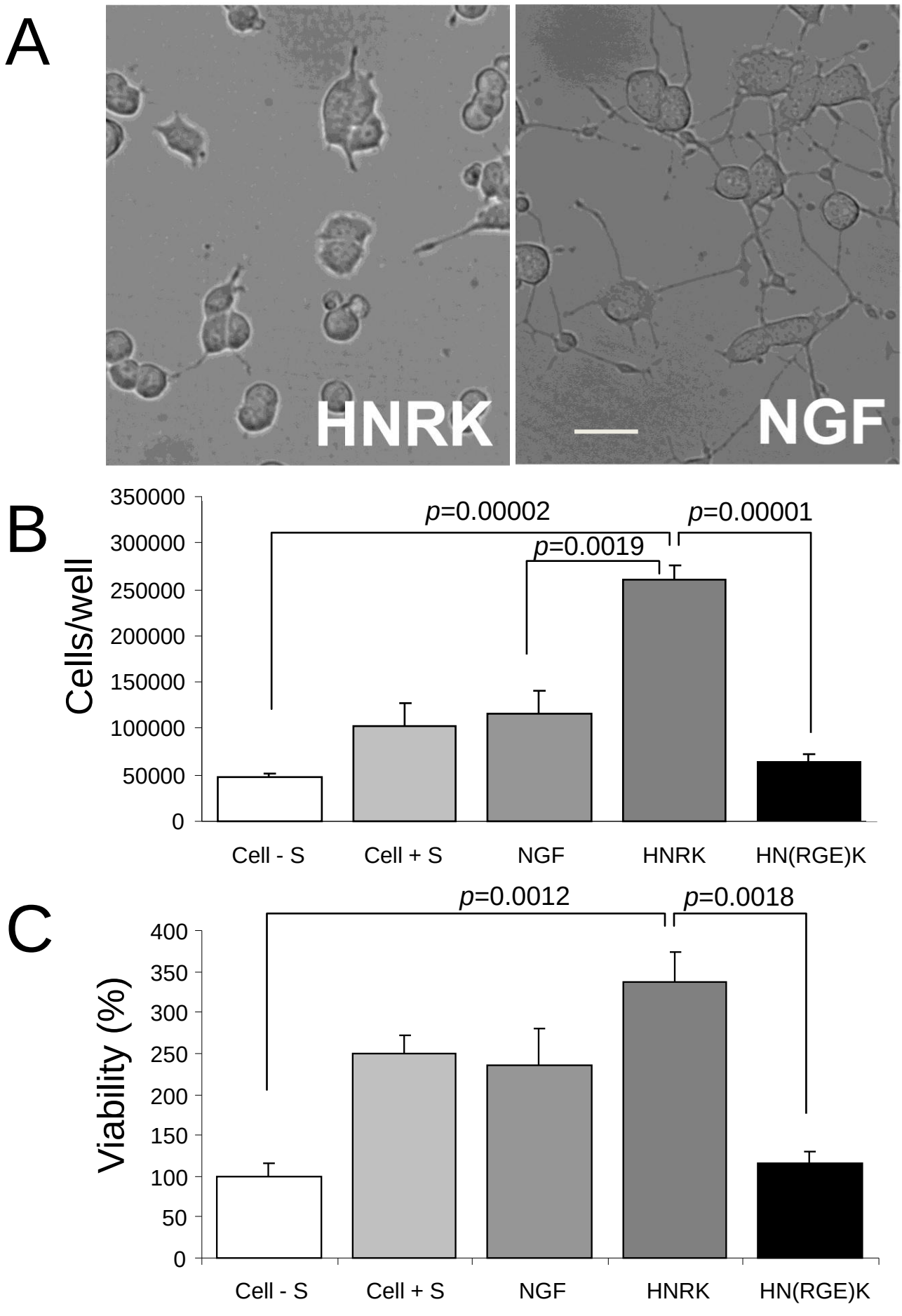
Figure 2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p=0.0019
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Cell + S
NGF
HNRK
HN(RGE)K
C
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300
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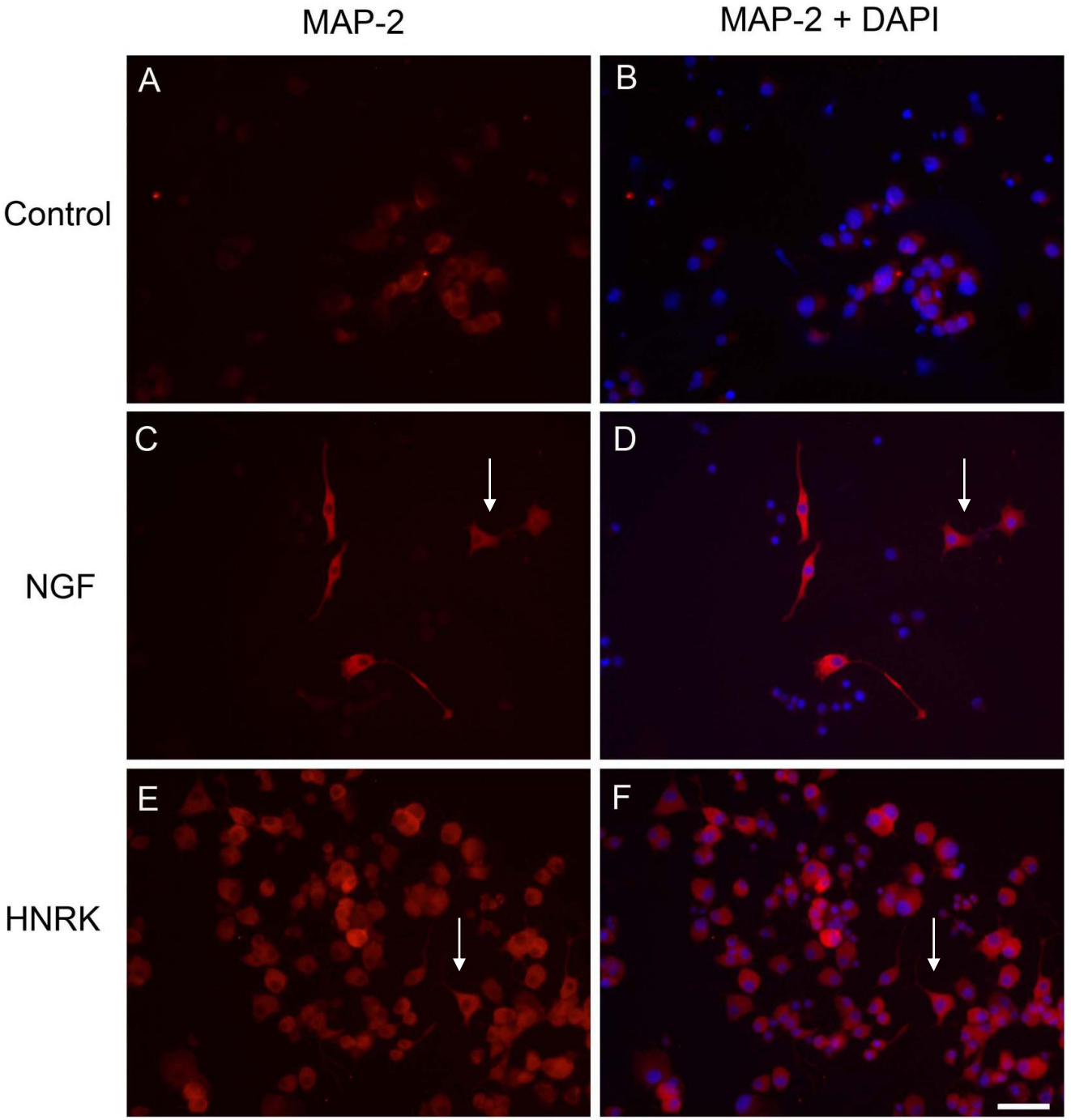
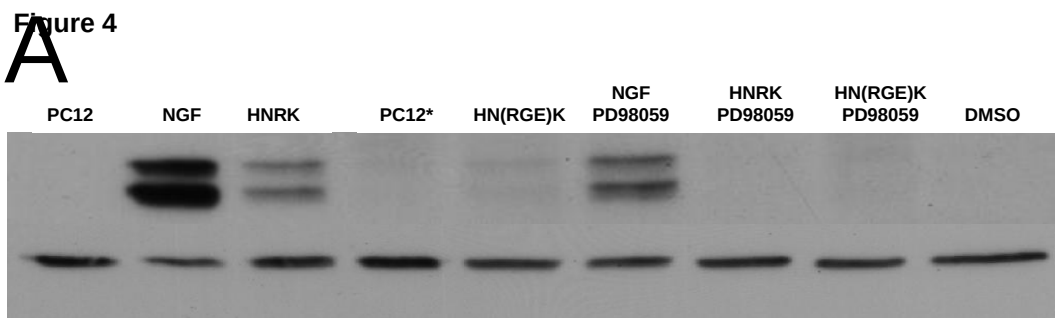


Figure 4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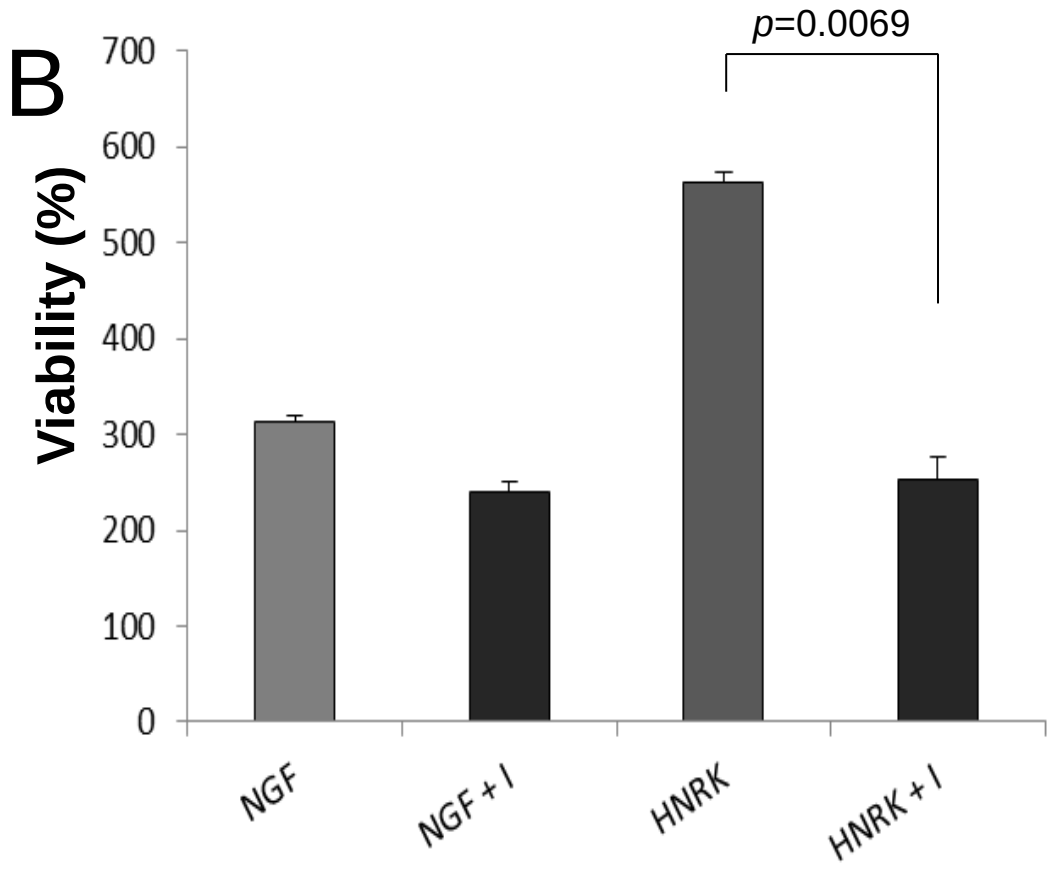
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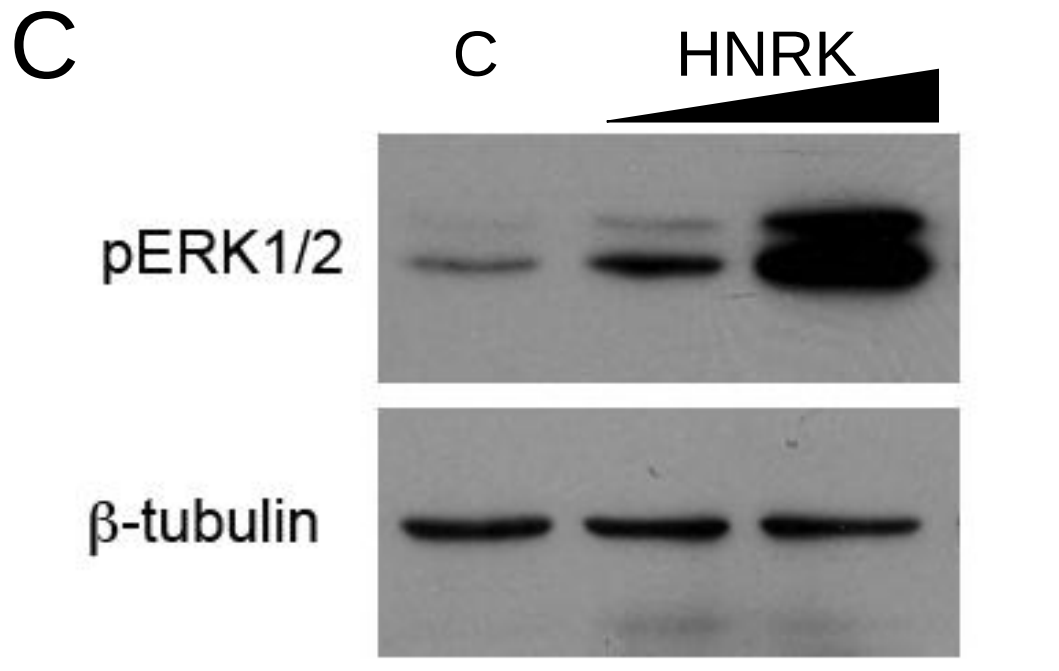
C
C
HNRK
pERK1/2
β-tubulin

Supplementary Material

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