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QUINOLINIC ACID PROMOTES THE ACTIVATION OF CULLIN3 AND ENHANCES NRF2 UBIQUITINATION IN THE NUCLEUS OF RAT STRIATAL CELLS

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Highlights

- Quinolinic acid (QUIN) induces nuclear expression of Cul3 in rat striatal cells
- QUIN increases NEDD8 immuno-reactivity in rat striatal cells
- Both events lead to the formation of Cul3-Keap1-Nrf2 complexes in the nuclei
- The Neddylation pathway could be a pharmacological target for neurologic diseases

Abstract: Quinolinic acid (QUIN) is a neurotoxin that induces oxidative stress. The Nrf2- signaling pathway is a key player in the protection against oxidative stress, and is regulated by the interaction of Nrf2 with the Keap1 protein, which triggers Nrf2 degradation via the proteasome throughout Cullin3 (Cul3) ubiquitin ligase. In this paper, we show that QUIN induces nuclear expression of Cul3 and overexpression of its activator Nedd8 in both ex vivo and in vivo models, leading to the formation of Cul3-Keap1-Nrf2 complexes, with concomitant increase of Nrf2 ubiquitination. These results reveal the neddylation pathway as a key player on QUIN toxicity.

Keywords: Quinolinic acid, cullin 3, nedd8, nrf2, neurodegeneration.

INTRODUCTION

Quinolinic Acid (QUIN) is a metabolite produced by the catabolism of tryptophan, via the Kynurenine pathway and is considered an endogenous excitotoxin [1]. It has been associated to neurologic disorders ranging from Alzheimer's disease to depression, and its levels are increased in the central nervous system (CNS) during the ageing process [2]. Production of QUIN has been proposed as one of the early key events in the chain of pathophysiological events that lead to impairment of cell function in the CNS [1]. Several ways in which QUIN can trigger the neuropathological process have been described, however the main mechanism is still unknown, but has been suggested that one of the major damaging pathways could be the formation of reactive oxygen species (ROS) [2, 3] given that the brain is highly vulnerable to oxidative stress, which increases with age; this could be due to a decrease in both detoxification capacity and protective cell pathways activity [4]. In young and healthy individuals, cellular accumulation of ROS is counteracted by an endogenous antioxidant response. The key player of this cytoprotective response is the nuclear erythroid 2-related factor 2 (Nrf2), which induces synthesis of detoxifying phase II enzymes, such as heme oxygenase 1 (HO-1) and gamma-glutamylcysteine synthetase (γ-GCS), among others [5, 6]. Nrf2 activity is regulated by its interaction with Kelch-like ECH-associated protein 1 (Keap1), a BTB-like protein that is required for Nrf2 anchoring to cytoplasm and for directing Nrf2 degradation by the proteasome system [7-9].

Former studies assumed that under basal conditions, Nrf2 binds to a dimer of the Keap1 protein in the cytoplasm, promoting interaction of the Cullin3 (Cul3)-based E3 ubiquitin ligase complex and Nrf2, which leads proteasomal degradation of Nrf2 [8, 10]. However, evidence suggests that Nrf2 may also be degraded in the nucleus, either by a proteasome-dependent mechanism or by another independent pathway [10-12]. Exposure to various agents may induce dissociation of Nrf2 from Keap1, and therefore, inhibiting its degradation (reviewed in [13]). Although the effects of Keap1-posttranslational modifications on Nrf2 regulation have been extensively explored, little is known about the influence of Cul3 modifications on the control of this response. Nevertheless, it could play a key role, given the relevance of the cullin protein

family in several biological processes [14-16].

The assembly and activity of all cullin-based ligases, including Cul3 protein, are regulated through reversible conjugation of a small ubiquitin-like or SUMO-like protein, known as NEDD8 (*Neural Precursor Cell Expressed Developmentally Down-Regulated 8*) [17]. Neddylation enables cullin-RING ligase (CRLs) multiprotein complexes, where cullins play a scaffold role, to promote ubiquitination of target proteins [16-19]. Due that the activity of CRLs is based on consecutive cycles of neddylation/dennedylation, which are regulated through a protein complex named COP9 (Constitutive photomorphogenesis 9) signalosome, that functions as an enzyme for dennedylation (reviewed in [16]), has been speculated that Cul3 inactivation by inhibiting neddylation could stabilize Nrf2. [10, 20]. In fact, we have previously reported that use of a specific neddylation inhibitor, MLN4924, allows nuclear accumulation of Nrf2 in cerebellar granule neurons (CGN), thus protecting it from the oxidative insult induced by hydrogen peroxide [21]. These findings suggest that neddylation may play a key role in regulating the oxidative stress response in neurons.

In this work, we studied the effect of toxic concentrations of QUIN on Cul3 expression and activity in the striatal cells of the rat brain. We found that QUIN induces accumulation of Cul3 and NEDD8 proteins in the nucleus of cells, both *ex vivo* and *in vivo*, leading to the formation of a Cul3-Keap1-Nrf2 complex in this subcellular compartment. This complex appears to increase the ubiquitination rate of Nrf2, suggesting that NEDD8 activates Cul3. Thus, we propose a new damage mechanism mediated by QUIN, which opens the possibility of new therapeutic approaches for pathologies where this pathway is involved.

MATERIALS AND METHODS

ANIMALS

Twelve Wistar male rats weighing 250-300 g were obtained from the National Institute of Neurology and Neurosurgery Manuel Velasco Suárez (INNNMVS) vivarium, under standard light and food conditions, and with unlimited access to purified water. All procedures were performed in accordance with Mexican standards for animal welfare, care, and experimentation (NOM-062-ZOO-1999), as well as international protocols. The INNNMVS Bioethics Committee approved all experiments.

STRIATAL SLICE PREPARATION AND TREATMENTS

350-400 µm coronal slices of the striatum were obtained from both hemispheres of fresh brains of rats, transferred to ice-cooled HEPES medium (pH 7.4), and then, recovered for 30 min at 37°C in Krebs-HEPES solution (124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 25 mM Na-HEPES, 1 mM $CaCl_2$ and 12 mM glucose) previously saturated with carbogen (95% v/v O_2 and 5% v/v CO_2). These slices were transferred to oxygenated Krebs-HEPES solution supplemented with vehicle or QUIN (100 µM) for 30 min and incubated at 37°C. At the end of this incubation, the slices were washed with PBS and frozen at -70°C until use.

SUBCELLULAR FRACTIONATION AND PROTEIN EXTRACTION FOR IMMUNOBLOT ANALYSIS

Nuclear and cytoplasmic proteins were obtained from slices, according to a reported protocol [22] and subsequently, western blot was performed. Fifty µg of proteins were separated by electrophoresis in a 10% SDS-PAGE gel (Bio-Rad Laboratories, Inc.), and then transferred into PVDF membranes

(Immobilon™-P, Millipore-Merk), which were incubated sequentially with primary antibodies: anti-Cul3 1:1000 (mouse monoclonal, Santa Cruz Biotechnology, sc-166054) and anti-NEDD8 1:1000 (mouse monoclonal, Santa Cruz Biotechnology, sc-373741), along with the anti-mouse IgG-peroxidase antibody (Sigma-Aldrich, Cat. A9044). For protein signal detection, Immobilon™-P (Millipore-Merk) and Kodak BioMax light films and reagents (Carestream, Health Inc.) were used. As a loading control, the membranes were incubated with the anti-GAPDH antibody (mouse monoclonal, Santa Cruz Biotechnology, sc-25778) for cytoplasmic extracts, or anti-histone 3 antibody (rabbit polyclonal, Sigma-Aldrich, Cat. H0164) for nuclear extracts standardization. Quantification of obtained bands was done with the ImageJ Program (rsb.info.nih.gov/ij/).

STEREOTAXIC STRIATAL INJURY WITH QUIN

To produce an *in vivo* experimental model of damage to the striatum, a lesion was induced by intrastriatal administration of 240 nmol of QUIN in eight rats, according to a previously published protocol [23]. In parallel, other eight rats with the same characteristics were administered with saline solution, as controls.

IMMUNOFLUORESCENCE

Forty-eight h after stereotactic surgery, three animals of each group were deeply anesthetized with sodium pentobarbital and perfused with 100 ml of 4% paraformaldehyde (PFA). Their brains were obtained and immediately placed in a 30% sucrose and 4% PAF solution in PBS for 3 days at 4°C. These brains were washed extensively in PBS, and then included into Tissue-tek® O.C.T. compound (Sakura Finetek) for sectioning in a cryostat (Microm HM 520, Microm International GmbH, Germany) mounted on slides Superfrost®/Plus (Fisherbrand™) and stored at -70°C until use.

Slides were incubated with the anti-Cul3 or anti-NEDD8 antibodies (1:100), for 8 h at 4°C, then washed and re-incubated with the respective secondary antibody conjugated to FITC (Sigma-Aldrich) and stained with DAPI (Sigma-Aldrich). After washing, the slides were mounted with fluorescence mounting medium (Dako, CA; USA), observed with the 40X objective using an Inverted Fluorescence microscope (Leica DM IL, Leica Microsystems, Germany) and finally captured and analyzed with the Leica IM1000 imager manager (Cambrige, UK).

CO-IMMUNOPRECIPITATION

Nuclear lysates

Five rats lesioned intrastriatally by QUIN (240 nmol) and five received saline solution (CT) by the same way were sacrificed after 48 h by decapitation. The striatum of each brain hemisphere was obtained, and those from the same experimental group (QUIN or CT) were pooled in a microcentrifuge tube and immediately frozen in liquid nitrogen. Then, striatal tissues were placed in a mortar and homogenized with the pestle, until a fine powder was obtained, which was stored at -70°C until processing.

Samples of CT and QUIN groups were rapidly thawed and resuspended by pipetting up and down several times in 1 ml of ice-cold PBS with a protease inhibitor cocktail (Sigma-Aldrich). The volume of each tube was separated into two equal parts in microcentrifuge tubes, which were labelled as "A" (CT and QUIN) and "B" (CT and QUIN). After centrifuging the four tubes for 10 min at 4,000 x g at 4°C, the supernatant was discarded. To obtain the nuclear extracts, "A" samples (CT and QUIN) were treated according to

the protocol described by Krämer and Keller [24] and "B" samples (CT and QUIN) were processed following the protocol described by Zhang et al. [25], which includes the use of N-ethylmaleimide (NEM) as inhibitor of deubiquitinases. For further details of these protocols see supplementary information.

Co-immunoprecipitation of Cul3-based complexes

Sepharose beads (Protein G Sepharose®, Fast flow; Sigma-Aldrich, Cat. P3296) were washed twice in PBS and then volume was restored with one volume of respective nuclei extraction buffer (50% suspension of sepharose). Nuclear lysates (500 µg of total proteins) were precleared and immunoprecipitated with the anti-Cul3 monoclonal antibody and 200 µl of resuspended beads. These immune complexes were washed 5 times with a washing buffer containing 50 mM tris-HCl pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, and 5 mM $MgCl_2$. A protease inhibitor cocktail was added and 5 mM N-ethylmaleimide (Sigma-Aldrich, cat. E3876) was added into the "B" tubes. Subsequently, the samples were migrated to a 10% SDS-PAGE gel, under mild reducing conditions based on the Blue-Native Polyacrylamide Gel Electrophoresis (BN-PAGE) technique, to promote the release of small amounts of Nrf2 protein from Cul3-Keap1-Nrf2 complexes (see supplementary information).

Immunoblot

Detection of Cul3-Keap1-Nrf2 complexes was evaluated by immunoblot analysis, using the anti-Nrf2 polyclonal antibody (C-20, Santa Cruz Biotechnology, sc-722) as was described above.

STATISTICAL ANALYSIS

Data was reported as the mean ± SD and analyzed using the t-student's test for comparison between groups on GraphPad Prism 5.0 (San Diego, CA, USA). The differences observed were considered statistically significant with values of $p < 0.05$.

RESULTS

QUIN ALTERS THE EXPRESSION OF PROTEINS CUL3 AND NEDD8 IN THE NUCLEUS OF STRIATAL CELLS

Changes in Cul3 activity due to the presence of QUIN were assessed by quantification of Cul3 and NEDD8 protein levels in the cytosol and nucleus of the cells obtained from the *ex vivo* model. In the case of control (CT) slices, Cul3 is found in cytosol, whereas in the nucleus it is virtually undetectable (Figures 1A-1C). Furthermore, the levels of NEDD8 protein expression in undamaged cells (CT) are very low in both compartments (Figures 1A, 1B and 1D). In contrast, incubation with QUIN promotes a notorious increase of Cul3 and NEDD8 levels in the nucleus of cells, without significant changes in the cytosol (Figures 1A-1D).

These results were confirmed *in vivo*, by using an indirect immunofluorescence technique. Figure 2A shows that in the striatum, the location of the Cul3 protein 48 h after the intrastriatal injection of saline solution for the CT group, is mainly cytoplasmic, whereas in the striatum of rats injured with QUIN, the Cul3 protein is found in the nucleus of most cells. In fact, three cell populations could be distinguished: i) cells in which Cul3 was located only in the nucleus, ii) another group with Cul3 located both in the nucleus and in the cytoplasm, and iii) cells with Cul3 located in the cytoplasm only (Figure 2B and 2C and supplementary material). It is possible that the presence of QUIN could mainly affect neurons, given that the Nrf2 factor is constitutively destabilized in this cellular type [2], whereas it is accumulated in astrocytes [26, 27]. Therefore, the few cells in

which Cul3 is not located in the nucleus after the treatment with QUIN may correspond to astrocytes or microglia cells.

Although changes in the levels of NEDD8 were determined in the striatal slices by Western blot, these levels are less significant than those of Cul3 (Figure 1D). A clear increase of fluorescence detection in the striatum in response to treatment with QUIN was observed for the *in vivo* model, and this event apparently involves all the cell types of the striatum (Figure 2D). This is consistent with studies that have reported overexpression of the NEDD8 protein in different nervous system disorders [28, 29].

QUIN PROMOTES THE FORMATION OF ACTIVE CUL3-KEAP1-NRF2 COMPLEXES IN THE NUCLEUS OF THE STRIATAL CELLS

Our findings suggest that changes of Cul3 and NEDD8 expression in the nucleus could lead to Cul3 activation in this compartment, possibly resulting in the *in situ* degradation of its substrate proteins, and Nrf2 factor could be one of the most relevant. To demonstrate this hypothesis, an assay of co-immunoprecipitation of nuclear proteins to detect Cul3-Keap1-Nrf2 complexes was performed, since previous studies have shown that these proteins can form a ternary complex in a proteasome-sensitive manner, which can be isolated by this technique [8,30].

Figure 3 shows a very notorious thick band with a high molecular weight (larger than 235 kDa) in the nuclear extracts obtained from the striatum of the rats lesioned with QUIN, which is not visible in the extracts of the control rats. Therefore, we propose that this shows the active Cul3-Keap1-Nrf2 complex, since activation of Cul3 requires the formation of a heterodimer formed by one neddylated Cul3 and one unneddylated Cul3 for subsequent degradation of its substrate proteins [31-33]. Consequently, the molecular weight of the active complex is higher than the one of the inactive complex; since the latter is composed by two Keap1 molecules and only one unneddylated Cul3, and only the active complex can contain the Nrf2 protein [20,31-33]. Bands with molecular weights of ~ 57 kDa and ~110 kDa can also be observed in Figure 3. Since these bands coincide with the expected molecular weights for Nrf2 [23,34], we can speculate that they correspond to Nrf2 that may have been released from the complex due to migration under mild reducing conditions. This seems to make sense, since additional bands produced by the treatment of nuclear lysates with N-ethylmaleimide (NEM), an inhibitor of deubiquitinating enzymes, was observed, possibly indicating polyubiquitination of Nrf2 (Figure 3, asterisks). Interestingly, it appears that nuclear ubiquitination of Nrf2 is a normal event, as previously suggested [11, 12], but is intensified by the presence of QUIN.

DISCUSSION

Nuclear levels of Nrf2 are known to rise rapidly in response to inducers such as tert-butylhydroquinone (tBHQ), or sulforaphane. QUIN also produces this effect [35], but our results indicate that presence of high concentrations of QUIN in the striatum over longer periods of time additionally leads to enhanced degradation of Nrf2, because QUIN increases also the formation of active Cul3-Keap1 complexes in the nucleus. In fact, the promoters of the genes encoding Keap1, Cul3, and Rbx contain ARE sites [36, 37], so an increase in Nrf2 levels eventually leads to an increase of the proteins involved in its degradation. This can explain the reduction of nuclear Nrf2 levels that was previously observed in rat brain striatal slices exposed to a prolonged incubation with QUIN [23].

Cul3 has been described as a cytoplasmic protein that does not travel between the cytoplasm and the nucleus, and therefore, activation of Keap1-Cul3 complex and subsequent ubiquitination of Nrf2 was assumed to be cytoplasmic, rather than nuclear [38]. Nevertheless, our findings coincide with those of Nguyen *et al.* [39] who present Nrf2 as a nuclear protein that requires translocation of Keap1 and Cul3, for its ubiquitination and degradation *in situ*. This is also supported by the fact that activation of Cul3 requires nuclear localization of the neddylation components [40, 41].

Cul3 activation has been shown to be dependent on the availability of NEDD8 molecules [32]. Furthermore, neddylation of Cul3 on lysine 712 is required for Nrf2 ubiquitination *in vivo* [20] and presence of the Keap1-Cul3-Roc1 complex causes polyubiquitination of Nrf2 *in vitro* [8]; therefore, our results seem to indicate that both an increase in levels of NEDD8 and changes in the Cul3 expression in the nucleus in presence of QUIN have as consequence, activation of the Cul3 complex *in situ* leading to an enhanced ubiquitination and degradation of Nrf2.

In a pioneering study [10], Kobayashi and collaborators suggested that modifications of Cul3 by NEDD8 could play an important role in the regulation of the antioxidant response. Additionally, it is known that diverse types of cellular stress can induce an increase in protein neddylation [42]. Thus, based on the results presented here, QUIN could trigger protein neddylation, producing alterations in Cullin-based complex formation. Besides Nrf2, other proteins involved in the regulation of diverse cellular processes and that could affect the health and performance of the neurons, have been described as substrates of Cul3 [43]; hence, alterations in its activity may lead to neuropathologic processes. Previous works have described that NEDD8 and the elements for its activation could be involved in the process of neurodegeneration [18, 29, 44], so the high immunoreactivity rate for NEDD8 in a wide variety of intracytoplasmic inclusions is not surprising. These inclusions are characteristic of several neurological diseases, including Lewy bodies in Parkinson's disease and dementia, systemic multiple atrophy, motor neuron disease, polyglutamine diseases, and neurofibrillary tangles in Alzheimer's disease [28, 29, 44-46]. As a neurotoxin, QUIN seems to induce the molecular mechanisms summarized in figure 4, where Cul3 neddylation could play an important role, and which is consistent with previous findings that demonstrate that an early effect of QUIN is the Nrf2 nuclear expression in absence of ROS [35].

CONCLUSION

Changes in the expression of Cul3 and NEDD8 proteins seem to be an important component of QUIN-induced neurotoxicity in the striatum. Our results suggest that deregulation of neddylation and cullin activity, particularly of Cul3, could be critical events in the mechanism of cellular damage in the brain, opening new possibilities for the treatment of these pathologies.

AUTHOR CONTRIBUTIONS

IGGH conceived the study, designed, and supervised the experiments, and analyzed data. ACAR performed the experiments and analyzed data. PDM performed the *in vivo* model of injury with QUIN. IGGH wrote the manuscript and PDM made manuscript revision.

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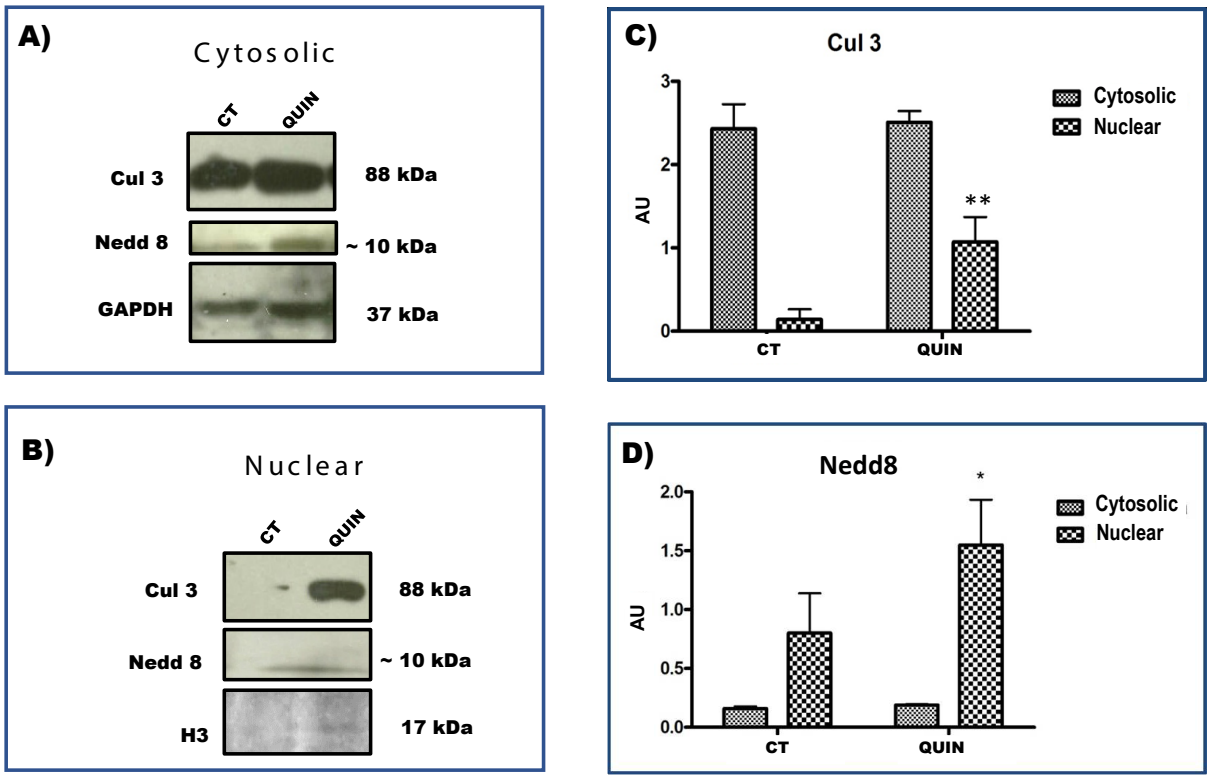
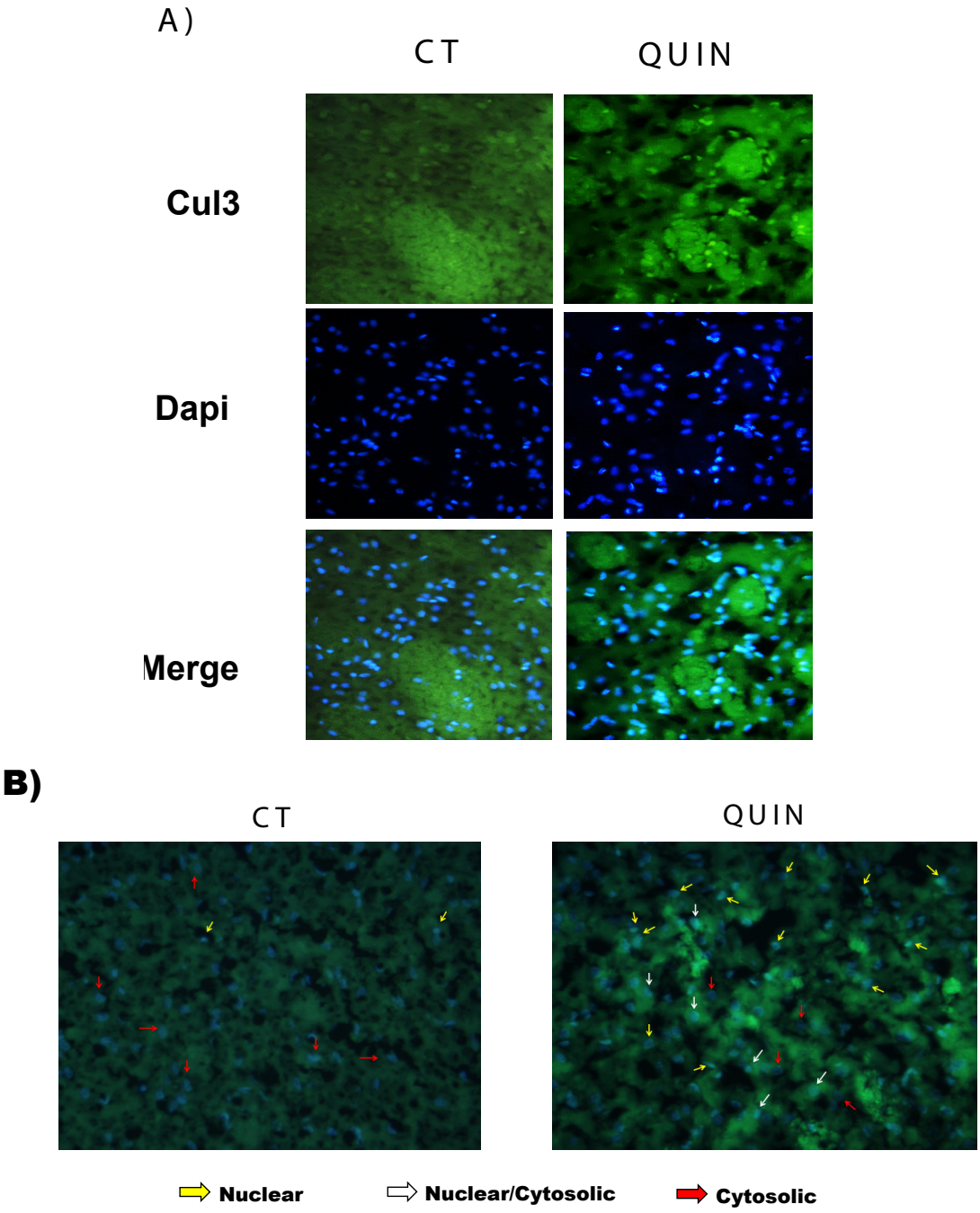


Figure 1. QUIN alters Cul3 and NEDD8 nuclear expression in rat striatal slices. Immunodetection of Cul3 (A) and NEDD8 (B) by Western blot in the coronal slices treated with QUIN (100 μM) or saline solution (CT). Immunodetection of GAPDH (A) and histone 3 (H3) (B) were used for normalization of the cytosolic and nuclear extracts, respectively. Densitometric analysis of Cul3 (C) and NEDD8 (D) expression levels in cytosolic and nuclear fractions of striatum are shown. These experiments were performed in triplicate using five slices per group in each experiment. Values are the mean ± SD. *p=0.0642 and **p=0.0079 vs CT group. AU = arbitrary units.


A)
CT
QUIN
Cul3
Dapi
Merge
B)
CT
QUIN
Nuclear
Nuclear/Cytosolic
Cytosolic

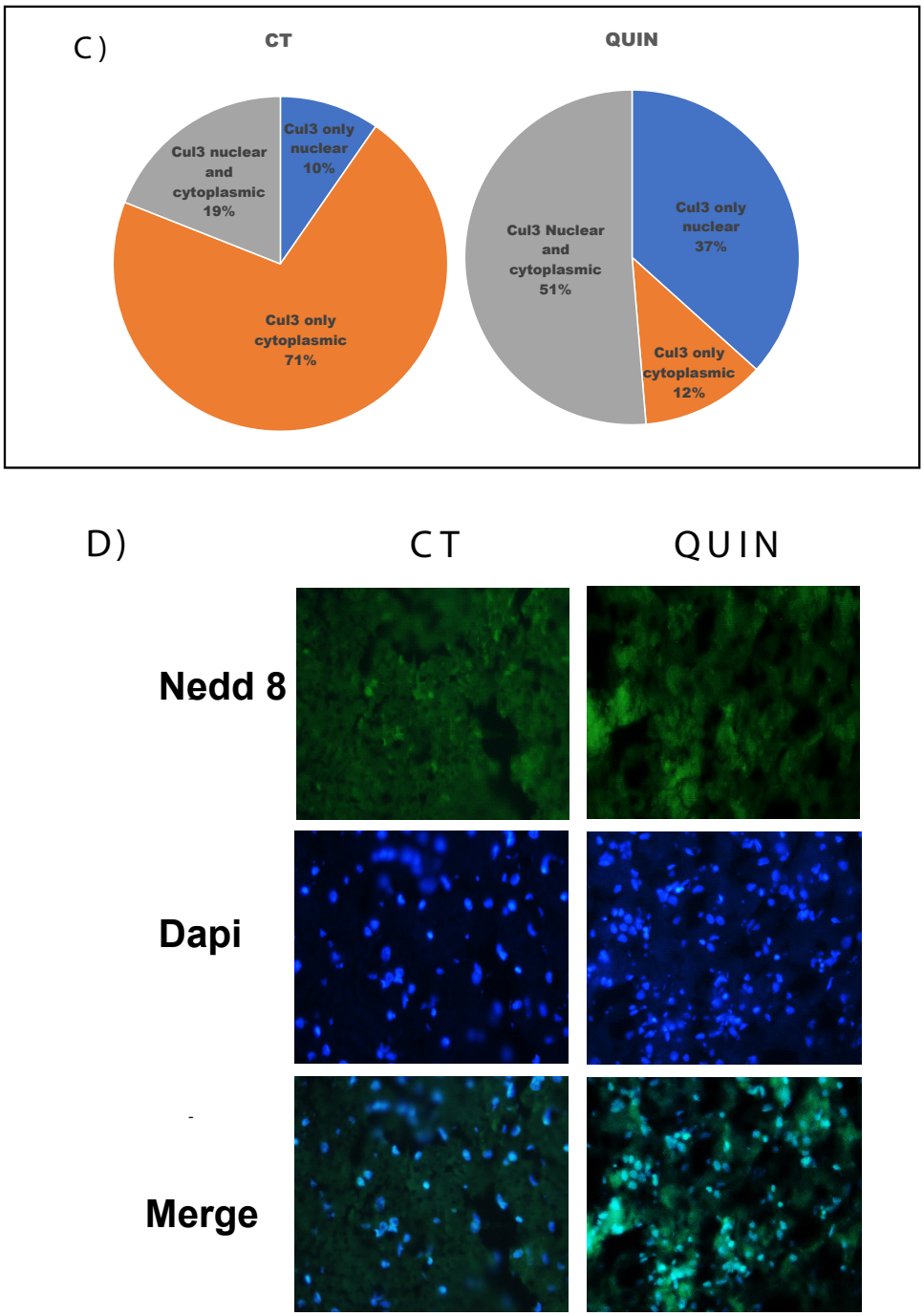


Figure 2. QUIN increases Cul3 and NEDD8 protein levels in rat striatum *in vivo.* (A) Indirect immunofluorescence in rat striatum sections (thickness 10 μm) incubated with anti-Cul3 monoclonal antibody and with a secondary antibody coupled to FITC (green). The nuclei were stained with DAPI (blue). (B) Three cell populations can be distinguished in the QUIN-treated striatum, in contrast with the control (CT) brains, where a minimal proportion of cells with nuclear Cul3 is observed. (C) Percentage of the cell populations observed in striatal slice preparations from brains of rat treated with QUIN or control (CT) animals. (D) Indirect immunofluorescence in rat striatum sections (10 μm) incubated with anti-NEDD8 monoclonal primary antibody and a secondary antibody coupled to FITC (green) and counterstained with DAPI (blue). A perfect overlap of NEDD8 with the nuclei of all cells is observed at 48 h post-treatment with QUIN.

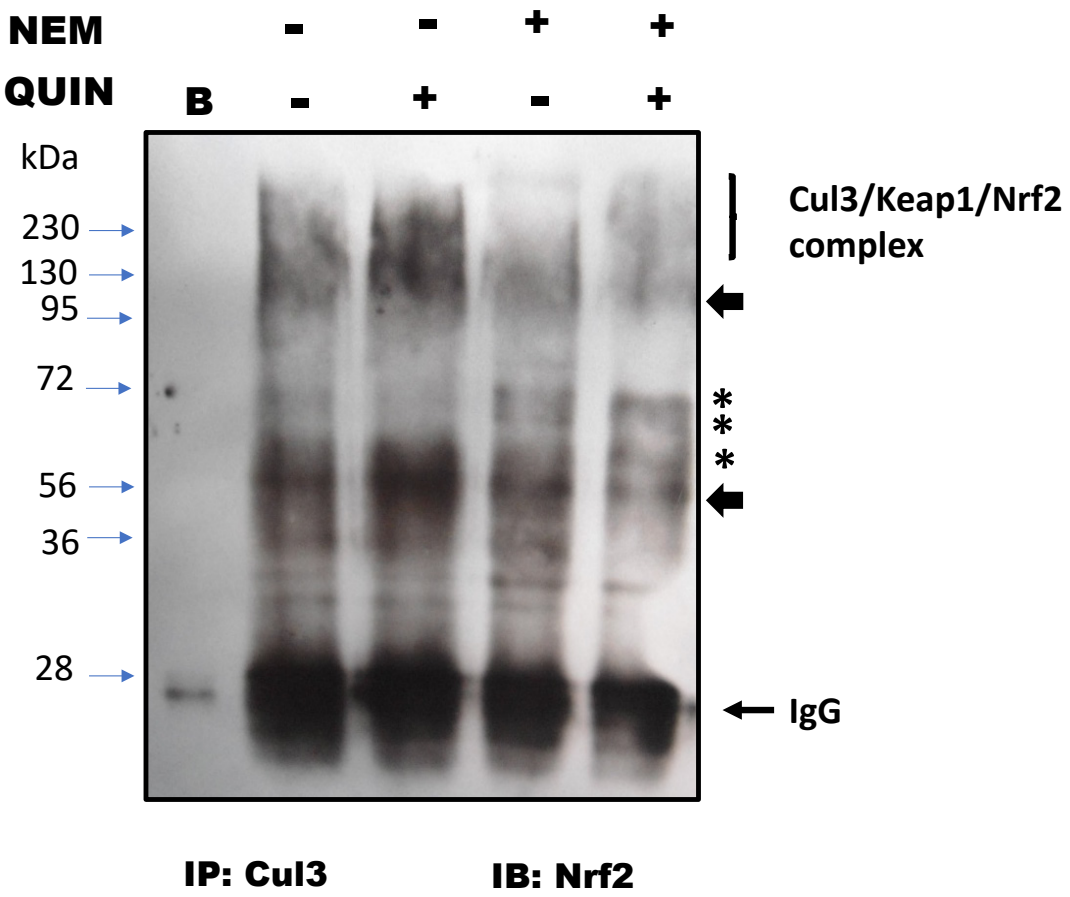


Figure 3. QUIN induces formation of Cul3-Keap1-Nrf2 complexes in the nucleus from rat striatal cells. Nuclear proteins of striatums of rats treated unilaterally by intrastriatal injection of saline solution (-) or QUIN (+) were extracted according to two different protocols. Hence, Cul3-Keap1-Nrf2 complexes can be seen as well as the bands with molecular weights corresponding to Nrf2 (thick arrows), and other bands that appear to indicate the polyubiquitination of Nrf2 (asterisks), whose intensity is greater in the samples of rats treated with QUIN (lines 2 and 4), indicating a larger amount of Nrf2. As control, sepharose beads were incubated in the same way as the samples but in the absence of nuclear extracts (lane B).

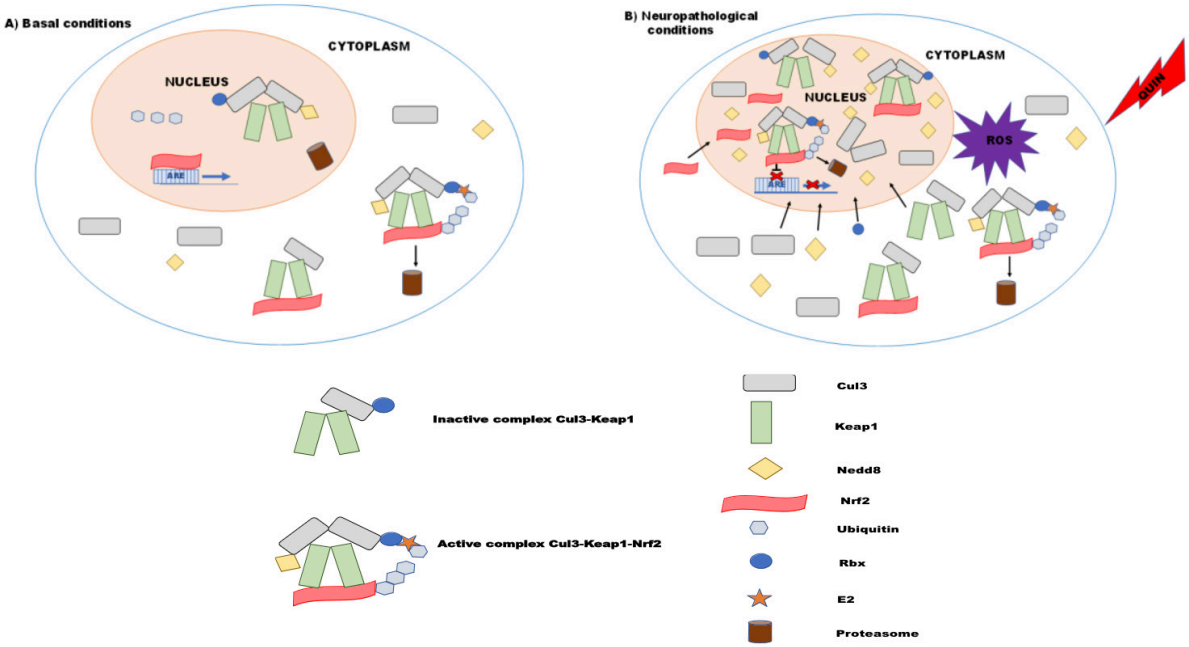


Figure 4. Model proposed for QUIN triggered neurodegeneration in striatal cells. (A) Under basal conditions (physiologic levels of QUIN or short-term exposure to high concentrations), Nrf2 nuclear levels rise steeply and absence of Cul3 in the nucleus restricts degradation of Nrf2 in this subcellular compartment, so Nrf2 can perform its function as an inducible transcription factor. (B) Prolonged exposure to toxic concentrations of QUIN causes synthesis of components of Nrf2 degradation such Cul3, Keap1 and Rbx1. The persistent presence of QUIN also raises NEDD8 levels in the nucleus, allowing the activation of Cul3-Keap1 complexes in this compartment. This leads to an increase in Nrf2 protein degradation, inhibiting its protective function, along with depletion of antioxidant enzymes and the concomitant elevation of ROS.

SUPPLEMENTARY MATERIAL

CO-INMUNOPRECIPITATION

Nuclear lysates

The Krämer and Keller protocol (1990), used to obtain the nuclear extracts from "A" tubes consisted of pellet resuspension in two volumes of buffer containing 10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT. The tissue was manually homogenized and centrifuged at 664 x g for 10 min. The supernatant (cytoplasm) was eliminated, and the second pellet was slowly mixed with 4 volumes of a buffer containing 20 mM HEPES-KOH (pH 7.9) buffer, 25% glycerol, 1.5 mM $MgCl_2$, 0.42 mM NaCl, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT. Samples were shaken on ice for 30 min and the supernatant was obtained by centrifugation at 17,949 x g for 15 min.

The Zhang *et al*. protocol (2004) used for obtention of nuclear extracts from "B" tubes consisted of pellet lysis in an ice-cold buffer containing 150 mM NaCl, 1 mM KH_2 PO_4 (pH 6.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 0.2 mM DTT, 5 mM N-ethylmaleimide and 0.3% triton-X100. After centrifugation, the isolated nuclei were resuspended in ice-cold nucleus extraction buffer (500 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 10% glycerol, 5 mM N-ethylmaleimide and protease inhibitor cocktail (Sigma-Aldrich) and shaken for 1 h at 4°C, and finally centrifuged to recover the supernatants, which were adjusted to a final concentration of 150 mM NaCl using the same buffer without NaCl.

CO-IMMUNOPRECIPITATION OF CUL3-BASED COMPLEXES

In order to detect Cul3-Keap1-Nrf2 complexes and the ubiquitinated Nrf2 belonging to them, we modified a conventional immunoprecipitation and immunoblot technique based on the principle of Blue-Native Polyacrylamide Gel Electrophoresis (BN-PAGE), that separates the components of a multiproteic complex by use of mild reducing conditions. Laemmli Buffer was substituted by SDS-PAGE denaturing buffer (10% glycerol, 2% SDS, 50 mM Tris pH 6.8, 0.002 % Bromophenol Blue, 50 mM dithiothreitol), and samples were heated 5 min at 70°C, to promote the release of small amounts of the Nrf2 protein from Cul3-Keap1-Nrf2 complexes.

QUANTIFICATION OF DIFFERENT CELL POPULATIONS EXPRESSING CUL3

Cells from a microscopic visual field of one section of the brain tissue for each rat (one field per slide), were analyzed using the manual cell counting mode of ImageJ Software. In total, three different visual fields corresponding to three different brains were analyzed in both CT and QUIN rats (n = 3). Below is an example of a visual field corresponding to a preparation of a brain treated with QUIN (Figure S1). Results of total counting are reported in table S1, and statistics are shown in table S2.

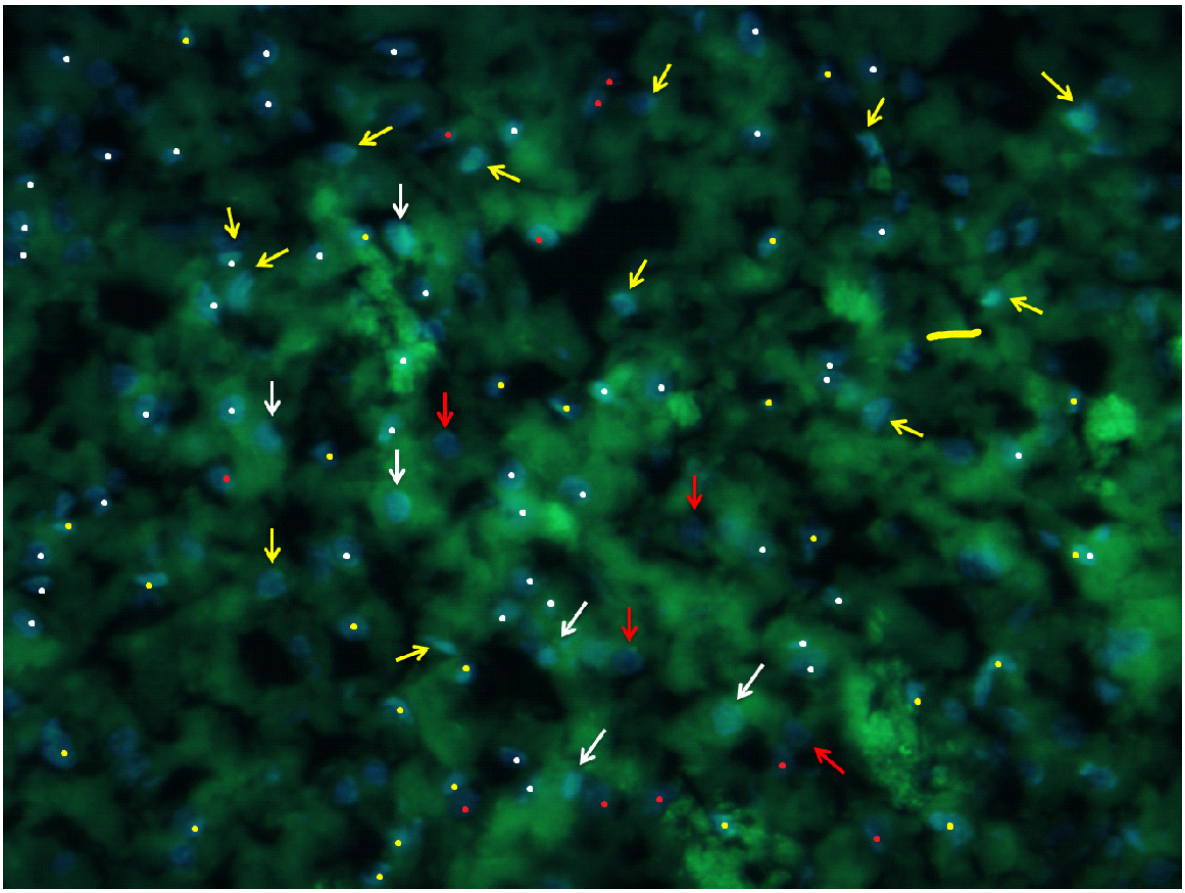


Figure S1. Visual field of a microscopic image of a brain tissue slide from one QUIN treated rat. One hundred cells were selected randomly (points and arrows), and classification and counting were carried out using the manual mode cell counting of ImageJ Software (https://imagej.nih.gov/ij/).

	Control			Quin		
	MEAN	**SD**	**n**	**MEAN**	**SD**	**n**
Nuclear	9,6666	2,0800	3	36,6666	2,5167	3
Cytosolic	71,3333	1,5275	3	12,3333	1,0125	3
Nucl & Cytos	19,3333	2,0100	3	51,3333	1,5275	3

Table S1.

	Significant?	**P value**	**Mean1**	**Mean2**	**Difference**	**SE of difference**	**t ratio**	**df**
Nuclear	*	0,000138039	9,6666	36,6666	-27,0	1,88504	14,3233	4,0
Cytosolic	*	< 0,0001	71,3333	12,3333	59,0	1,05408	56,2892	4,0
Nucl & Cytos	*	< 0,0001	19,3333	51,3333	-32.0	1,45296	22,2534	4,0

Table S2.

Statistical significance was determined using the Holm-Sidak method, with alpha=5.000% by means of a Multiple t Test in GraphPadPrism software.