

## Localization and Modulation of NEDD8 Protein in the Human Placenta

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**Abstract.** *Background:* Neural precursor cell-expressed, developmentally down-regulated-8 (NEDD8) is a 76-amino-acid ubiquitin-like polypeptide. NEDD8 affects the signaling of various molecules but the major cellular target proteins are cullins. The neddylation process is correlated closely with apoptosis, cell-cycle regulation, embryogenesis and development. *Aim:* The purpose of the present work was to investigate NEDD8 distribution and expression in the human placenta during gestation. *Materials and Methods:* A total of 30 samples, 15 chorionic villous samples from first trimester and 15 from full-term placentae, were used for the immunohistochemical analysis of NEDD8 expression. The gestation period ranged from 5 to 40 weeks. *Results:* NEDD8 was highly expressed in the cytotrophoblast of the first trimester of gestation, whereas in the third trimester, it was localized in the endothelial cells and stroma of placental villi. *Conclusion:* Our results suggest that NEDD8 may play an important role in the control of proliferation and differentiation of human placenta throughout pregnancy.

Neural precursor cell-expressed, developmentally down-regulated-8 (NEDD8) known as Rub1 in yeast is an 76-amino-acid, 6-kDa, ubiquitin-like polypeptide sharing 58% identity and 80% similarity respect to ubiquitin (1-3). NEDD8 is conservatively expressed in most eukaryotes or most, if not all, tissues suggesting its important function in

eukaryotic cells (4). It is a member of the ubiquitin-like protein superfamily (5) and shares a very similar three-dimensional structure with ubiquitin (6, 7). NEDD8 is first synthesized as a 81-amino-acid precursor and then it is processed at the five conserved C-terminal 5 amino acids following a Gly-Gly dipeptide by the hydrolase activity of de-neddylating enzymes (1, 8, 9).

Changes in NEDD8 abundance affect the signaling of various molecules, including auxin in plants (10), interferon regulatory factor-3 (IRF3) (11), murine double minute-2 (MDM2) (12), and p21 (13), but the major cellular target proteins for NEDD8 are cullins that form cullin-RING (Really Interesting New Gene) ubiquitin ligases (CRL) involving several hundred members (3, 14). Oved (15) expanded the functional spectrum of NEDD8 to include one of the best-studied ubiquitin-dependent processes, namely trafficking of membrane proteins (3, 16, 17). During cellular differentiation and for correct cell homeostasis, it is required that several proteins are degraded in a spatially and temporally controlled manner (18). Principal actors in this regulatory mechanism are the ubiquitins (19). Ubiquitination consists of large protein complexes, also known as Skp1, Cullin, F-box protein (SCF), divided into different groups called ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3). NEDD8 is able to regulate SCF by ligation in neddylation, increasing E3 activity and in part its affinity for E2 (18). Neddylation involves a three-step cascade: ATP-dependent activation catalyzed by the NEDD8 E1 enzyme NEDD8 activating enzyme E1 subunit 1-ubiquitin-activating enzyme 3 (NAE1-UBA3), transfer to a NEDD8 E2 conjugating enzyme ubiquitin-conjugating enzyme C12 (UBC12) and ubiquitin-conjugating enzyme E2F (UBE2F), the conjugation to a lysine residue in a target protein *via* an isopeptide bond mediated by NEDD8 E3 ligase (14, 20). Neddylation stimulates ubiquitination of several key proteins such as p27

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and inhibitor of kappa B ( $I\kappa\beta$ ) (21), and regulates the cytoplasmic level of  $\beta$ -catenin (18, 22). Neddylation results in the activation of CRLs by promoting the recruitment of the ubiquitin charged E2 enzyme and allowing its correct positioning relative to the substrate (14, 23-25).

A general elevation in the level of NEDD8 conjugation has been observed in oral squamous cell carcinoma cell lines, where NEDD8 pathway inhibition reduced cell proliferation (26). Several newly-discovered NEDD8 substrates are established tumor suppressors or oncoproteins, including von Hippel-Lindau protein, p53, p73, breast cancer-associated gene 3 and MDM2 (9). Moreover, the general inhibitor of the NEDD8 pathway, MLN4924, was shown to possess tumor-inhibiting properties (9, 27).

It has been demonstrated that the neddylation process is closely correlated with apoptosis, cell-cycle regulation, embryogenesis and development (5).

The NEDD8 system is essential for cell-cycle progression, including endoreduplication, which is an unusual mode of cell cycle that results in duplication of the chromosome without intervening mitosis (22, 28, 29). Mitosis and endoreduplication share common mechanisms, such as fluctuation of cyclin-dependent kinases (CDKs) activity (30).

On the basis of the role of neddylation in the cell cycle, we decided to investigate NEDD8 expression in the human placenta throughout gestation by immunohistochemistry.

## Materials and Methods

**Samples.** Human placental samples were obtained with informed consent from patients undergoing surgery such as cesarean section for normal placenta and uterine evacuation for normal chorionic villi. A total of 30 samples, 15 chorionic villous samples from first trimester and 15 from full-term placentae, were used and the gestation period ranged from 5 to 40 weeks. The collected specimens were immediately fixed in formalin for immunohistochemistry and immunofluorescence. Representative sections of each specimen were stained with hematoxylin-eosin and examined by a pathologist to confirm histological preservation of the microanatomic structure. The absence of any placental pathology was confirmed by microscopic observation.

**Immunohistochemistry and immunofluorescence.** Immunohistochemistry was carried out essentially as described previously (31, 32). Briefly, all sections were deparaffinized in xylene, rehydrated through a graded alcohol series and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and for antiserum dilution. Tissue sections were quenched sequentially in 3% hydrogen peroxide and blocked with PBS-6% non-fat dry milk (BioRad, Milan, Italy) for 1 h at room temperature. Slides then were incubated at 4°C overnight with an affinity-purified goat polyclonal immune serum raised against NEDD8 (sc-5480, Santa Cruz, CA, USA) at a 1:100 dilution and then with diluted anti-goat biotinylated antibody (Vector Laboratories, CA, USA) for immunohistochemistry or with anti-goat fluorescein isothiocyanate antibody (BioFX Laboratories, MD, USA) for immunofluorescence. All the slides then were processed by the ABC method (Vector Laboratories, CA, USA) for 30 min at room temperature. Diaminobenzidine (Vector

Laboratories, CA, USA) was used as the final chromogen and hematoxylin was used as the nuclear counterstain. Negative controls for each tissue section were prepared by substituting the primary antiserum with the respective pre-immune serum. All samples were processed under the same conditions.

For each specimen, an HSCORE value was derived by summing the percentages of cells/areas that stained at each intensity and multiplying that by the weighted intensity of the staining. Thus the maximum score was 360. An average of 22 fields was observed for each tissue by three observers at different times and the average score was used.

All values are expressed as the mean  $\pm$  standard error of mean (SEM) and differences were compared by one-way analysis of variance (ANOVA). *p*-Values less than 0.05 were considered significant.

**Classification of local subtypes of extravillous trophoblast (EVT).** EVT was classified into two morphological phenotypes (33): (a) the proliferative phenotype is represented by one to several compact layers of cells, which are attached to each other; (b) the invasive phenotype comprises the later post-proliferative EVT that no longer forms intracellular junctions. They become separated from each other and spread from the cell column into the surrounding basal plate and into the endometrium. The two different phenotypes can be easily discriminated by their topographical relations.

## Results

The expression and localization of NEDD8 in the human placenta throughout gestation was performed by immunohistochemistry and immunofluorescence using goat polyclonal antiserum. We observed a clear modulation of NEDD8 expression during pregnancy, with a high level of expression during the first trimester of gestation and a very weak level of expression during the third trimester. Specifically, in the first trimester human placenta, NEDD8 was expressed at high levels almost exclusively in the proliferative layer of cytotrophoblastic cells (Figure 1a). NEDD8 was particularly distributed in the cytoplasm of cytotrophoblast (Figure 1b). In the third trimester of gestation, NEDD8 expression strongly decreased until reaching a very weak expression level. It was localized almost exclusively in the stroma of placental villi and in the cytoplasm of endothelial cells lining blood vessels (Figure 1c).

In placentae of the third trimester of gestation, we also investigated NEDD8 expression in EVTs. NEDD8 was expressed at a very high level in the cytoplasm and in some nuclei of both proliferative and invasive EVTs (Figure 1d). In order to investigate NEDD8 localization deeply in the human placenta, we performed an immunofluorescence technique. We have shown that in the first trimester of gestation, NEDD8 is expressed in the cytotrophoblast and localized in the perinuclear region of the cells (Figure 1e). Figure 2 shows the peculiar expression pattern of NEDD8 during pregnancy as determined by immunohistochemistry analysis. Specifically, we observed a decrease in NEDD8 expression from the first to the third trimester of gestation.

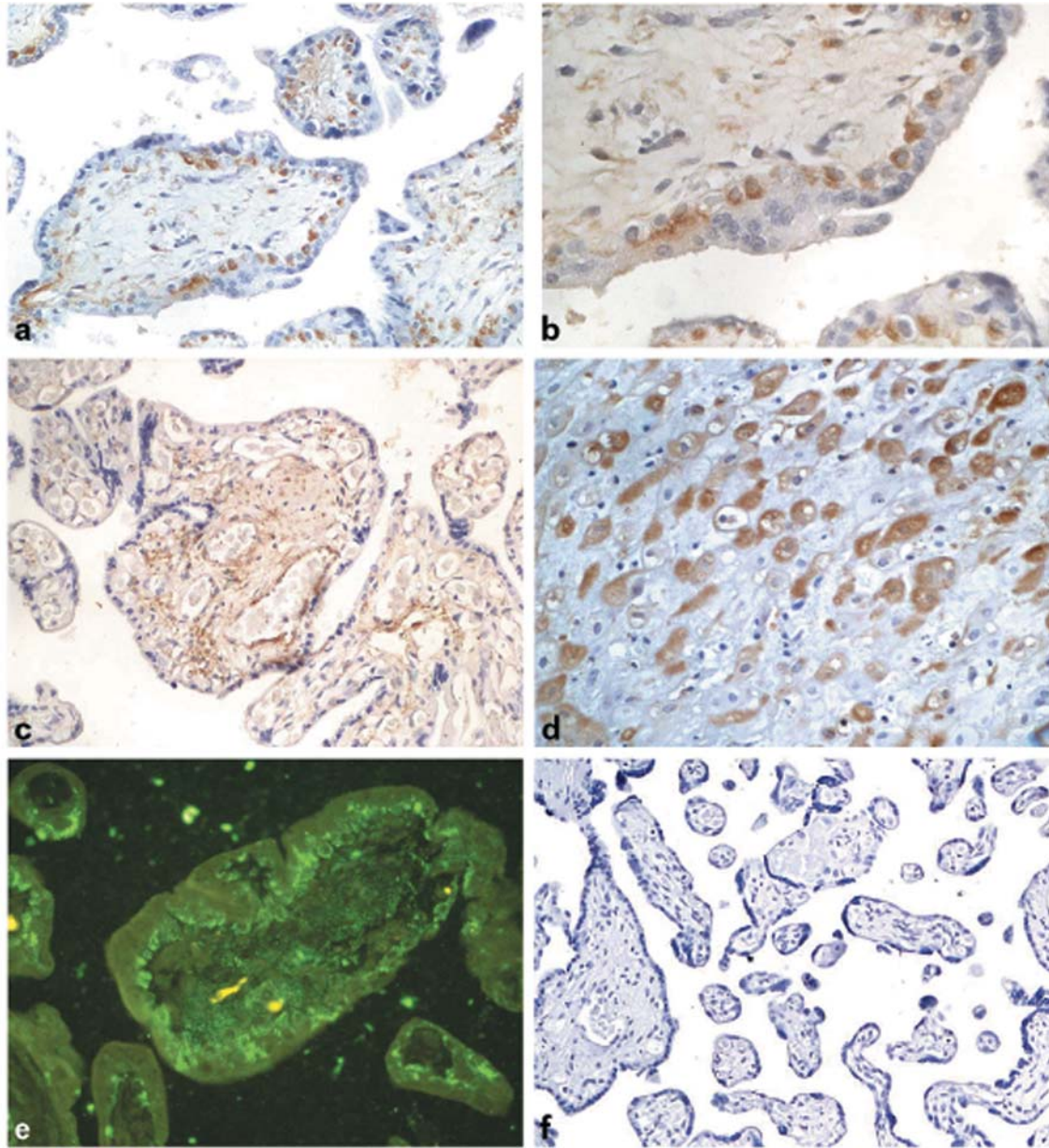


Figure 1. a: Strong Neural precursor cell-expressed, developmentally down-regulated-8 (NEDD8) expression distributed in the cytotrophoblasts of placental villi from the first trimester of gestation,  $\times 300$ . b: Higher magnification shows the perinuclear distribution of NEDD8 in the cytotrophoblasts during the first trimester,  $\times 640$ . c: NEDD8 expression in the stroma of placental villi and in the cytoplasm of endothelial cells during the first trimester of gestation,  $\times 300$ . d: High expression of NEDD8 in all extravillous trophoblasts in the third trimester of gestation,  $\times 640$ . e: Immunofluorescence analysis of NEDD8 localization in cytotrophoblasts in the first trimester,  $\times 300$ . f: Representative negative control of the third trimester of gestation,  $\times 150$ .

## Discussion

NEDD8 protein was found to be expressed in a wide variety of human tissues and was found to be particularly present in germ cells, epithelial cells, endocrine cells and peripheral ganglion cells (8). NEDD8 has an essential role in the regulation of protein degradation pathways, a mechanism

involved in cell-cycle regulation and differentiation. The NEDD8 system is also essential for cell-cycle progression and development of mammalian embryos (22). It has been demonstrated that NEDD8 overexpression interferes with physiological  $\beta$ -catenin turnover, suggesting a new possible target of neddylation (18). Moreover, the NEDD8 conjugation pathway is able to regulate the turnover of p27,



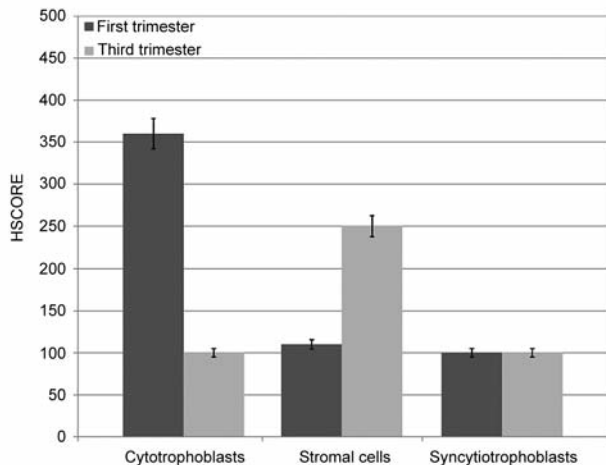


Figure 2. Gestational time course of Neural precursor cell-expressed, developmentally down-regulated-8 (NEDD8) expression in human placenta during pregnancy. NEDD8 expression in cytotrophoblasts, stromal cells and syncytiotrophoblasts during the first and the third trimesters of gestation is shown. Vertical lines indicate the show S.E.M.

interfering with withdrawal from the cell cycle (18, 21). The regulation of the NEDD8 conjugation pathway contributes to carcinogenesis. In many carcinoma cell lines, levels of NEDD8 conjugation were up-regulated (34), probably allowing for uncontrolled proliferation of malignant cells (5).

The human placenta is a complex organ that is composed of maternal and fetal material and plays an integral role not only in the supply of nutrients to the fetus but also in the maintenance of pregnancy (33, 35). The correct functioning of human placenta is due to the fine balance between proliferation, differentiation and invasion of trophoblastic cells. For this reason, human placenta is often compared to tumors. Definitive placental villi are formed by three distinct types of trophoblast cells: mononucleated cytotrophoblast forming an inner proliferative layer, multinucleated syncytiotrophoblast forming an outer non-proliferative layer, and by EVT (33). The EVTs of cell columns located proximally to the villous stroma have been identified as proliferating cells, whereas EVTs located distally from the villous stroma exhibit invasive characteristics and are no longer proliferative (33, 35). EVT invasion is exquisitely regulated by paracrine, autocrine, and juxtacrine mechanisms, involving several factors such as proteases and proto-oncogene expression (36-38).

The NEDD8 system is essential for both the mitotic and endoreduplicative cell cycles. The latter cycle occurs in several mammalian tissues, such as trophoblastic giant cells, hepatocytes and megakaryocytes (28, 38), and is involved in cell differentiation, cell expansion and resistance to DNA-damaging agents (22, 39, 40).

In the light of the role of NEDD8 in the cell cycle, we investigated its expression in human placenta where delicate regulation of cell vitality is essential for gestation. We demonstrated that NEDD8 expression decreased during pregnancy, from a high level of expression during the first trimester to a very weak signal in the third trimester. Intriguingly, we observed that in the first trimester, NEDD8 was localized almost exclusively in the cytoplasm, in a perinuclear region of cytotrophoblasts, the proliferative compartment of placental villi. Moreover, NEDD8 expression was high in EVTs, which are proliferative. It has been demonstrated that the loss of the NEDD8 system leads to selective apoptosis of the inner cell mass which forms the future embryonic ectoderm, probably inducing impaired replication or repair of DNA (22). Our results for NEDD8 expression in the human placenta are in agreement with previous results on regulation of apoptosis in the human placenta. We previously demonstrated that apoptosis increases from the first to the third trimester of gestation (41, 42), and that alteration of this pathway led to several placental pathologies and to abortion (35, 43). Furthermore, we observed that in the third trimester of gestation NEDD8 was localized in the stroma and in the endothelial cells lining blood vessels inside placental villi, suggesting a control in differentiation program necessary for the correct functioning of this organ. Localization and spatio-temporal distribution of NEDD8 in the human placenta are important enough to assign it a fundamental role in the control of proliferative and differentiation events of the placenta during pregnancy.

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