# Copper Dependent Modulation of α-Synuclein Phosphorylation in Differentiated SH-SY5Y Neuroblastoma Cells

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Presented by: Naliany & Yonika
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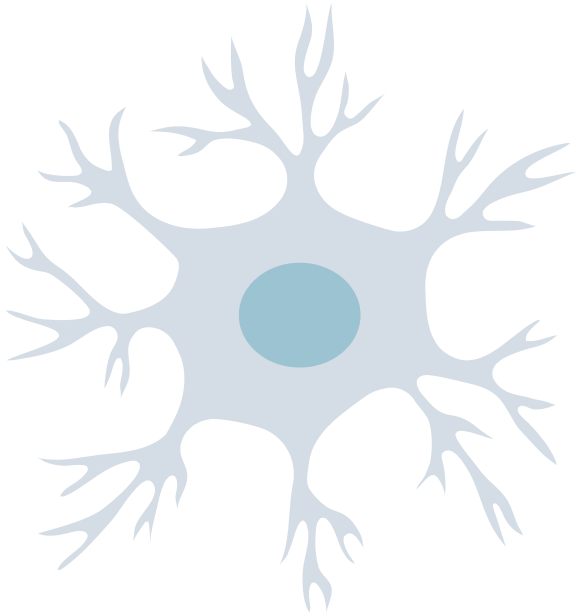
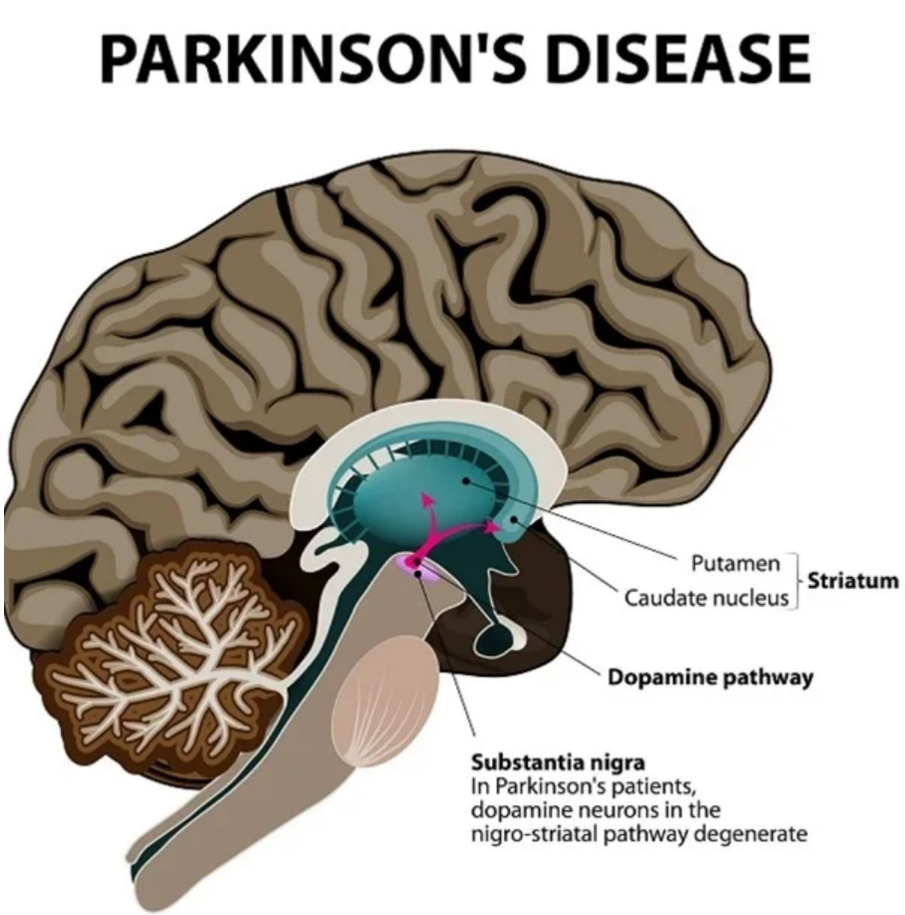
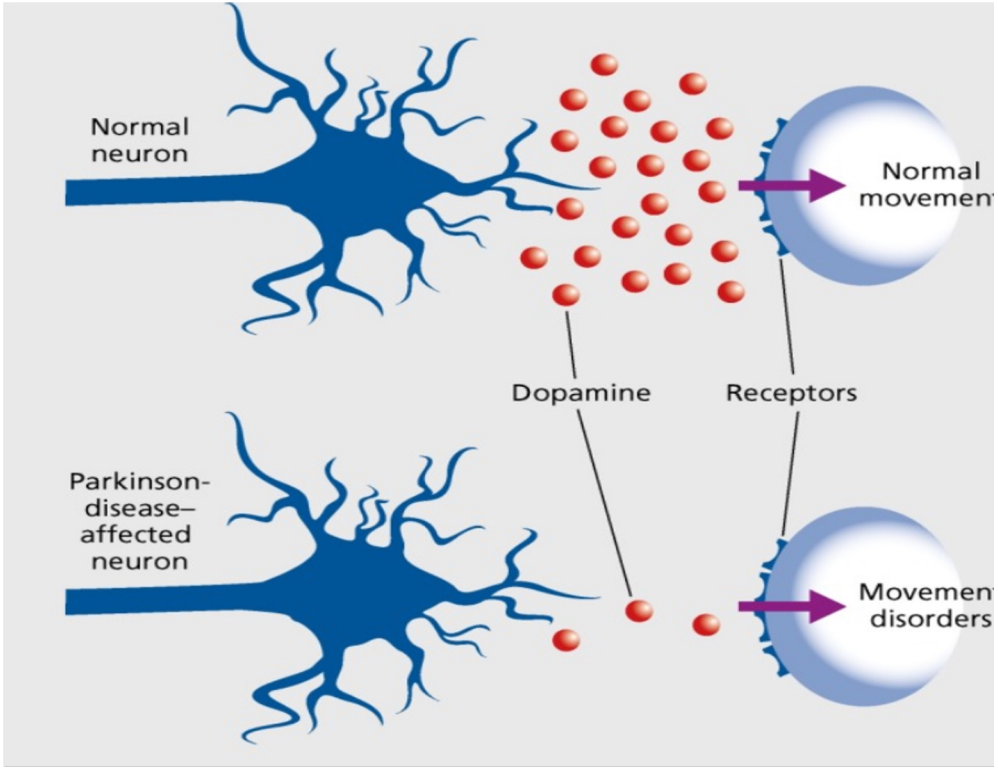


# Parkinson’s Disease (PD)

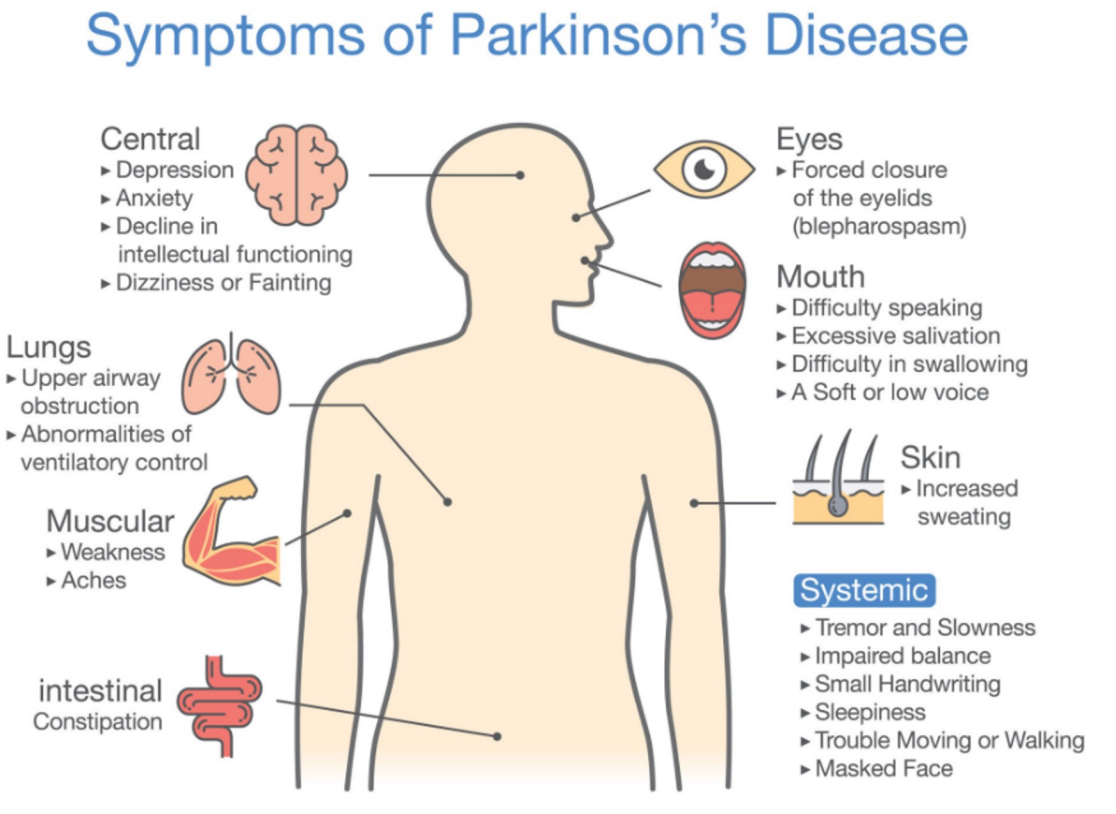



- Second most common
- Loss of cells in the substantia nigra of the brain - an area responsible for the production of dopamine (DA)
- DA- chemical messenger that transmits signal between regions of brain to coordinate activity

# Parkinson’s Disease (PD)



# PD symptoms




**Motor Symptoms:**

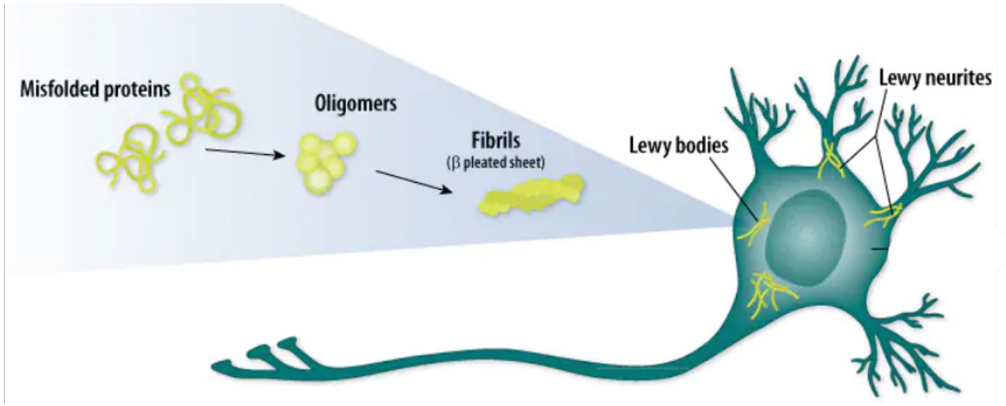
- Rigidity
- Tremors
- Delayed movement
- Poor balance

**Non-Motor Symptoms:**

- Sleep disturbances
- Urinary dysfunction
- Constipation
- Swallowing problems
- Mood disorders
- Cognitive deficits

# Pathogenesis

- The precise mechanism underlying the pathogenesis of PD is not yet understood.
- Accumulating evidence suggests that soluble α-synuclein aggregates, known as oligomers, play a significant role in the PD.
- Accumulation of α-synuclein leads to intra neuronal inclusion named lewy bodies.
- How Lewy bodies are specifically related to the progression of the disease is not known.



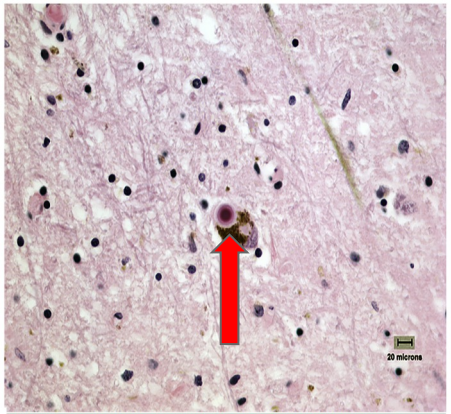
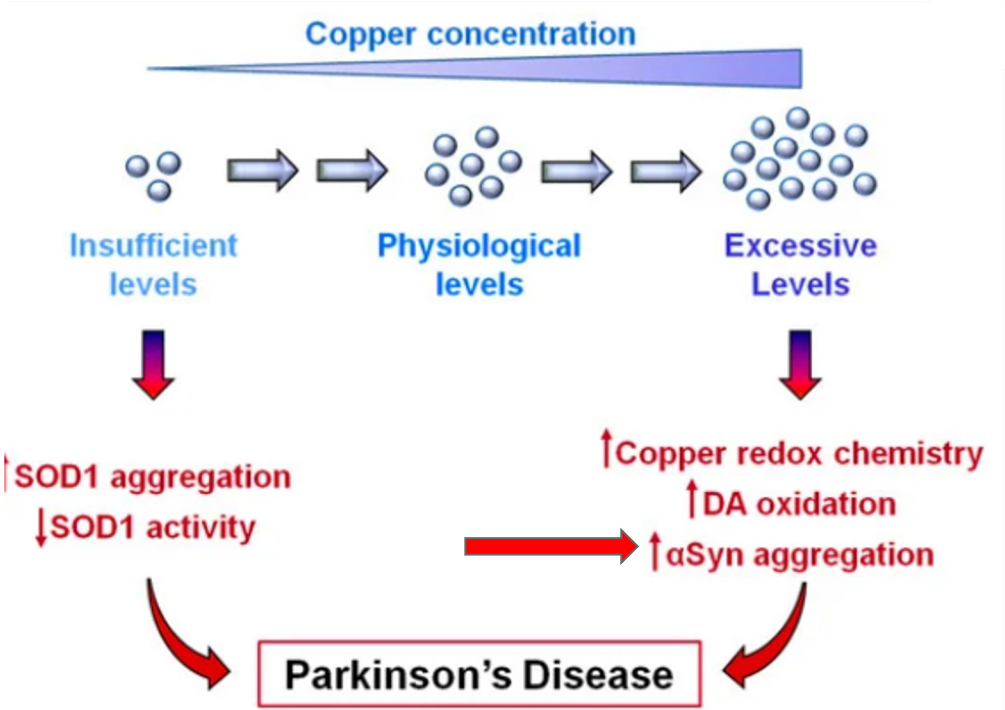




**Figure** Lewy body, kindly provided by Dr. Mel Feany, Harvard Medical School.

# Metals and PD?




- Metals such as manganese, lead, iron, and copper have been reported to play a role/considered as risk factors in the development of PD.
- Considering the numerous cellular pathways that require the presence of copper, it is not surprising that failures in the homeostatic processes leading to either copper excess or deficit can lead to severe disorders.

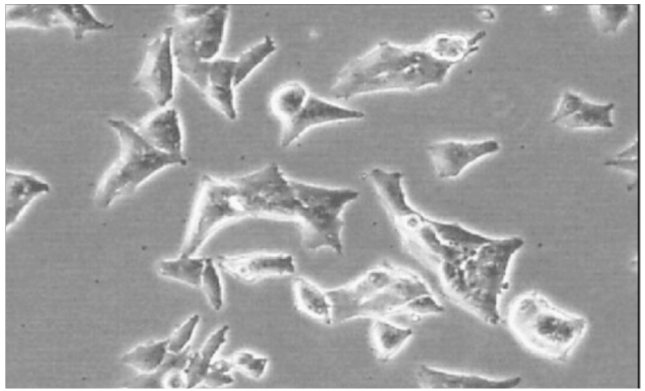
How does copper play a role in the developmental stages of PD?

**Hypothesis**

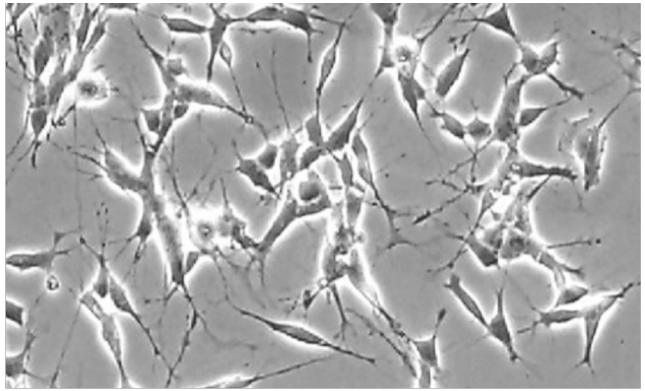
Copper primarily leads to accumulation of phosphorylated synuclein, an initial event in the onset of PD.

# PD model

Undifferentiated



Differentiated



- SH-SY5Y cells
- Neuroblastoma cell line
- Posses dopaminergic features
- Undifferentiated cells- low levels of dopaminergic enzymes, continuous mitosis- not a property of neurons
- Differentiated cells- more neuronal like, not dividing, high levels of neuronal markers.
- To differentiate them in to neuronal like retinoic acid and brain-derived neurotrophic factor are widely used
- Rotenone- widely used to create parkinsons *in vitro* and *in vivo*.

# Methods

- MTT dye for cell viability assay
- Copper content was analyzed by atomic absorption
- Western blot for protein analysis
- Reverse transcription-PCR for mRNA analysis
- Fluorescent dyes to analyze oxidative stress and mitochondrial membrane potential

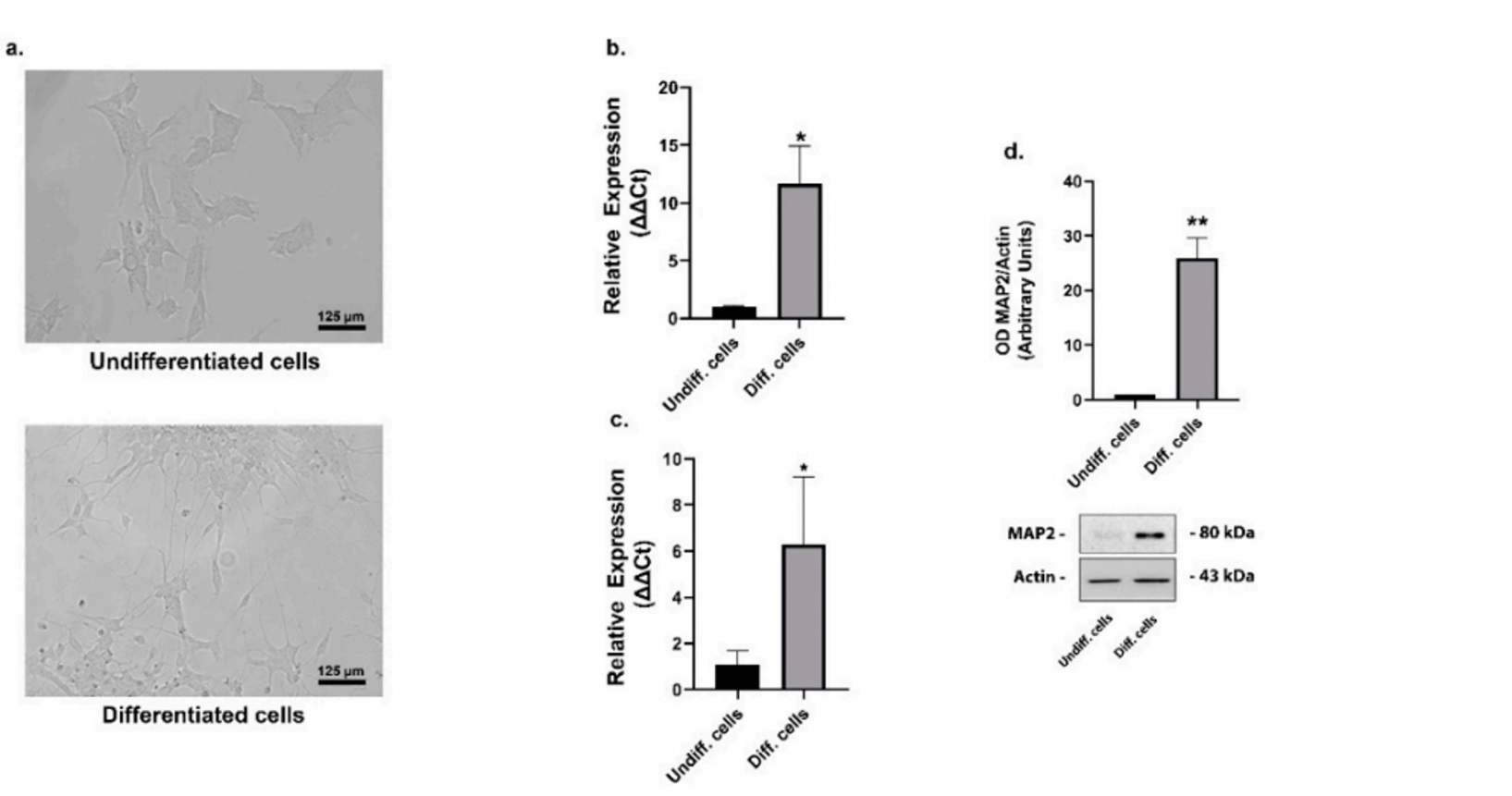



**Figure 1:** SH-SY5Y cells differentiation. Representative phase contrast images of cellular morphology of SH-SY5Y cells before (top left) and after (bottom left) the differentiation protocol. Morphology of differentiated cells showed prominent neurites outgrowth (a). Relative expression of the neuronal markers TH mRNA (b) and synaptophysin (c) significantly increased in differentiated cells (means ± SD of three independent experiments). Representative immunoblots of the neuronal marker MAP2 (d) in undifferentiated and differentiated cells. Densitometric analysis of bands represented means ± SD of three independent experiments (right). β-actin was used as loading control (c). The results were presented as means ± standard deviation of three independent experiments; values for differentiated cells were compared to undifferentiated cells by one-way ANOVA following Tukey test * $p < 0.0332$ ** $p < 0.0021$.

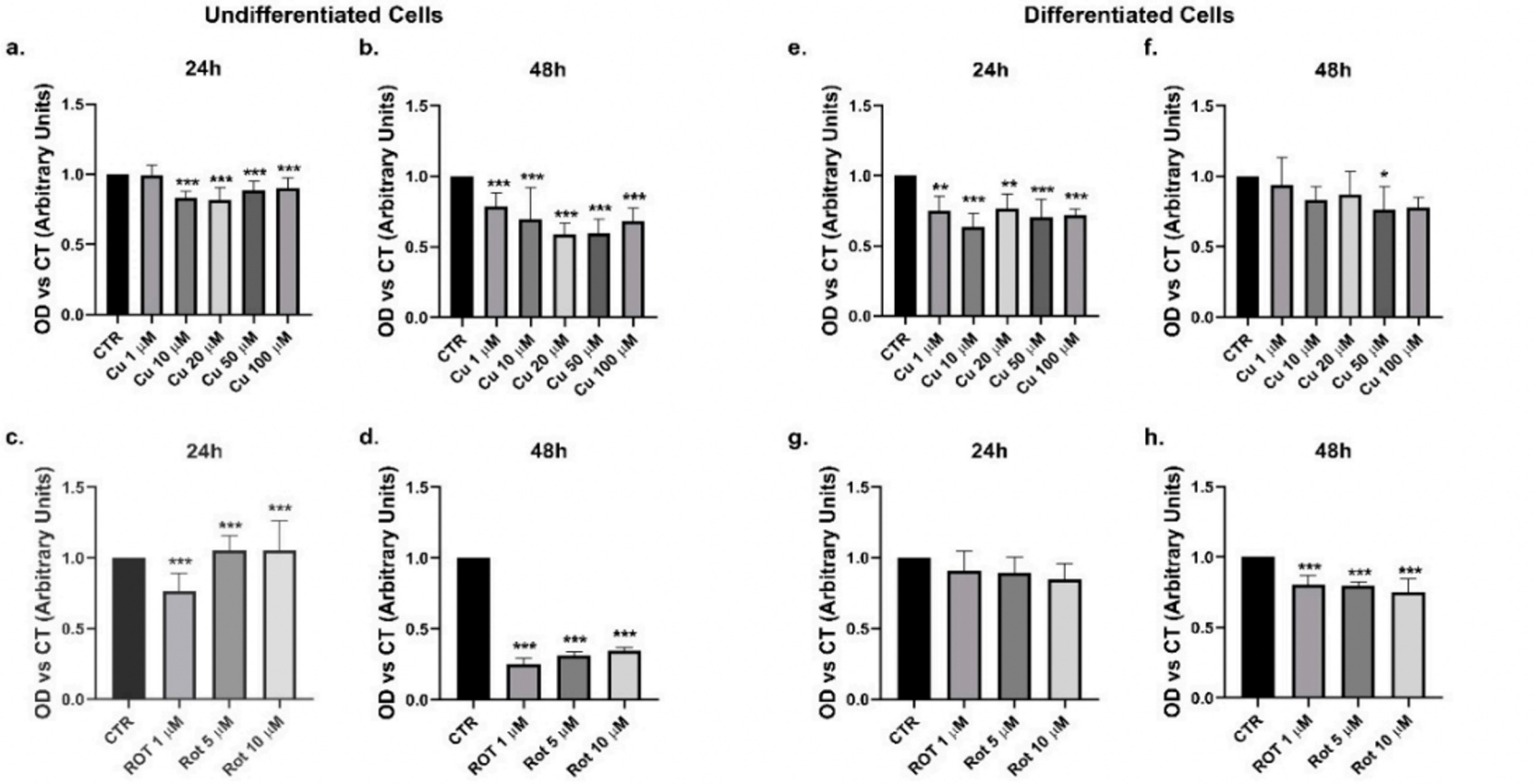



**Figure 2**: Effects of copper on cell viability (relative MTT assay). Undifferentiated SH-SY5Y cells were treated with the indicated concentrations of copper or rotenone for 24 h (a,c) and 48 h (b,d). The same treatments were performed in differentiated cells, also in this case for 24 h (e–g) and 48 h (f–h). The results are presented as means ± SD of seven replicates; values were compared with untreated cells (CTR) by one-way ANOVA following Tukey test * $p < 0.0332$ ** $p < 0.0021$ *** $p < 0.0002$, compared to untreated cells (CTR).

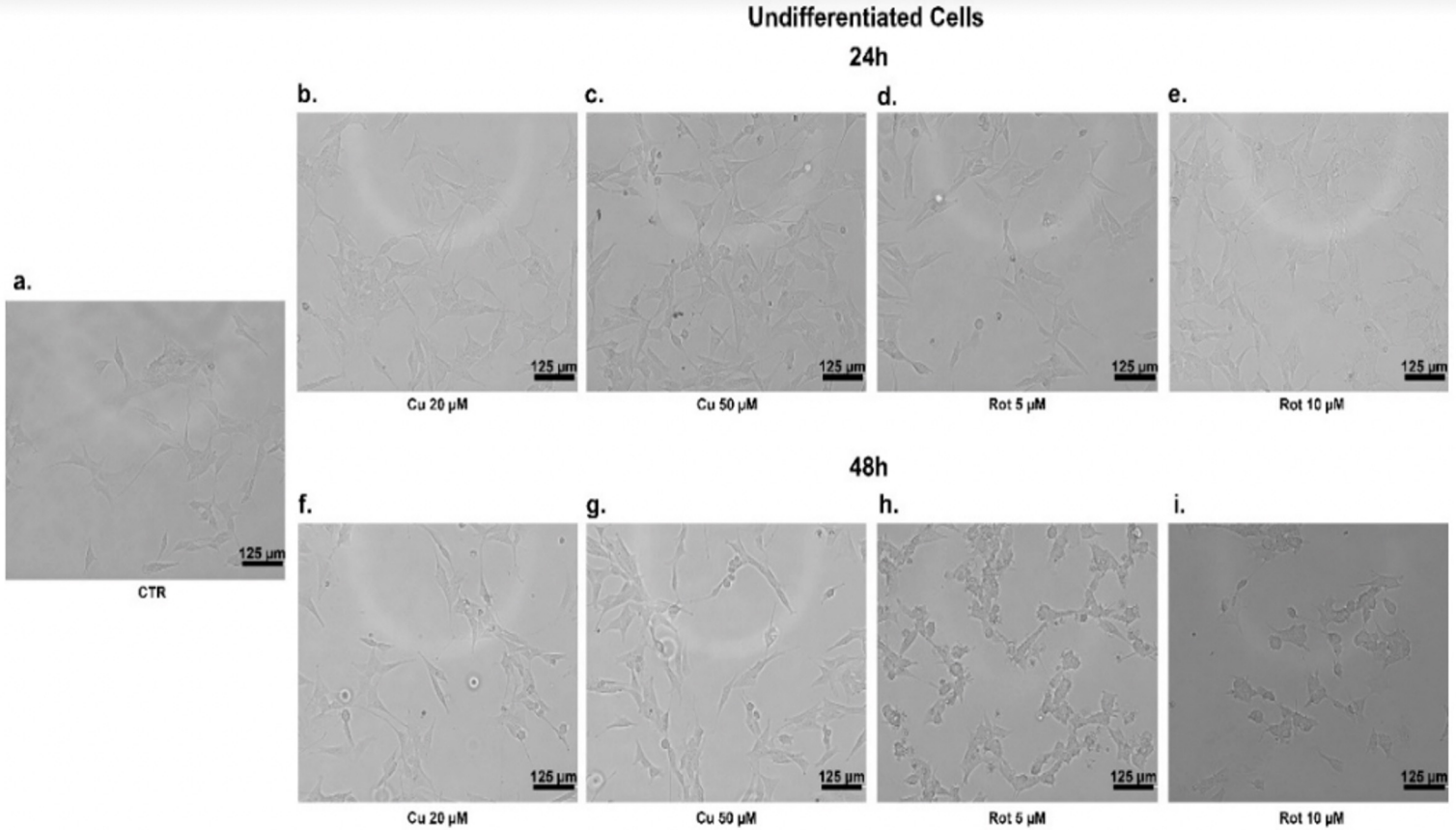



**Figure 3**: SH-SY5Y undifferentiated cells treatments. Phase contrast images of cellular morphology of SH-SY5Y undifferentiated cells after copper (b,c,f,g) and rotenone (d,e,h,i) treatments for 24 and 48 h. The morphological alteration in cells treated with rotenone for 48 h is noticeable, especially compared with the untreated cells on the left (a).

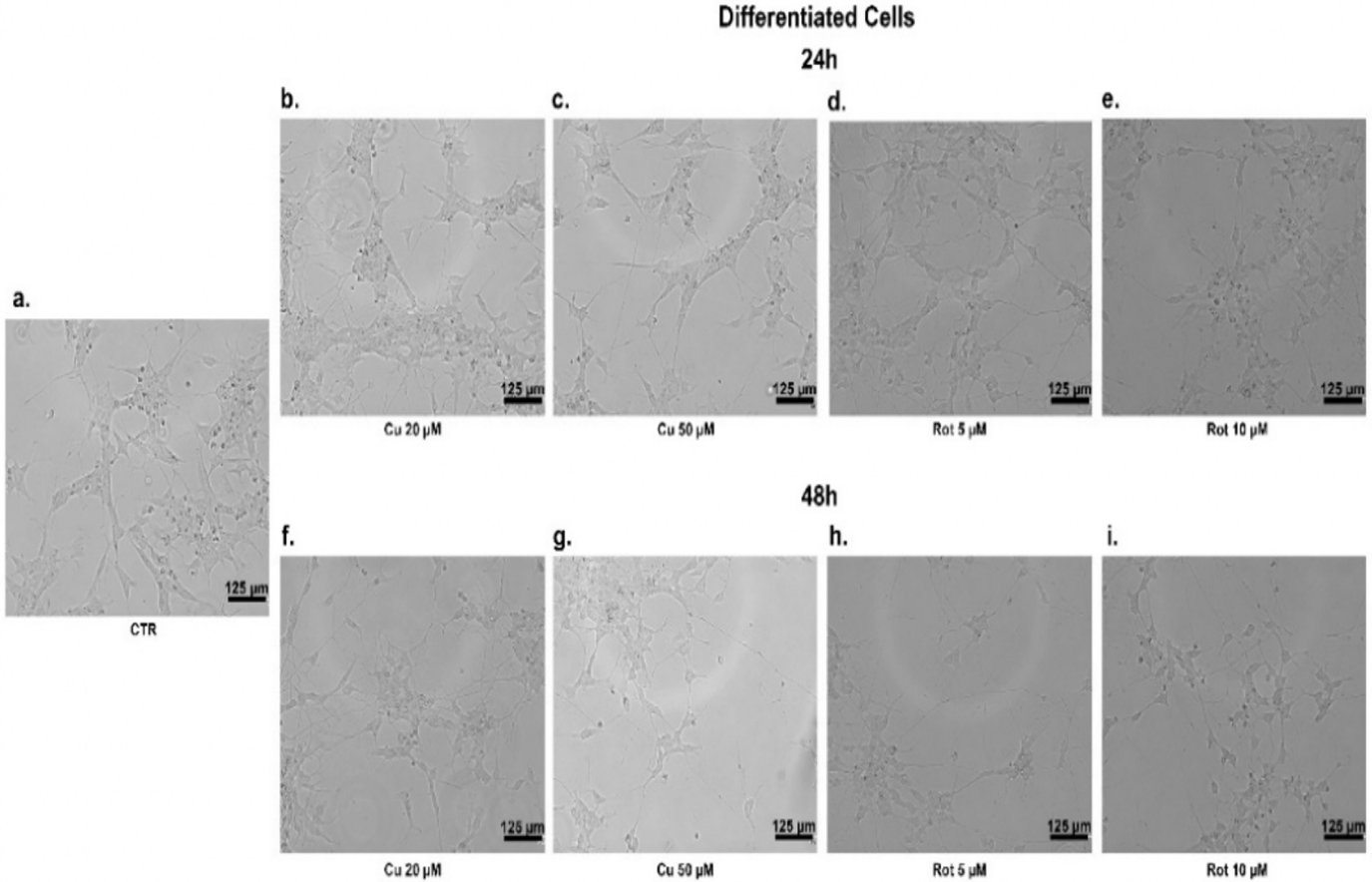



**Figure 4:** SH-SY5Y differentiated cells treatments. Phase contrast images of cellular morphology of SH-SY5Y differentiated cells after copper (b,c,f,g) and rotenone (d,e,h,i) treatments for 24 and 48 h. Untreated cells on the left (a).

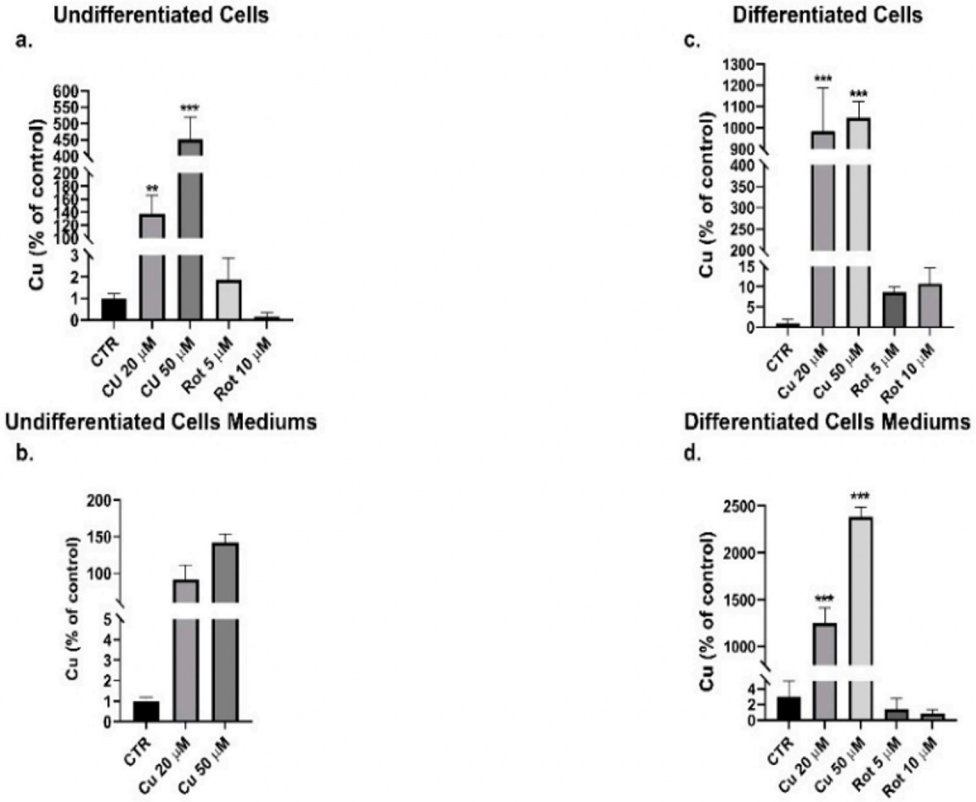
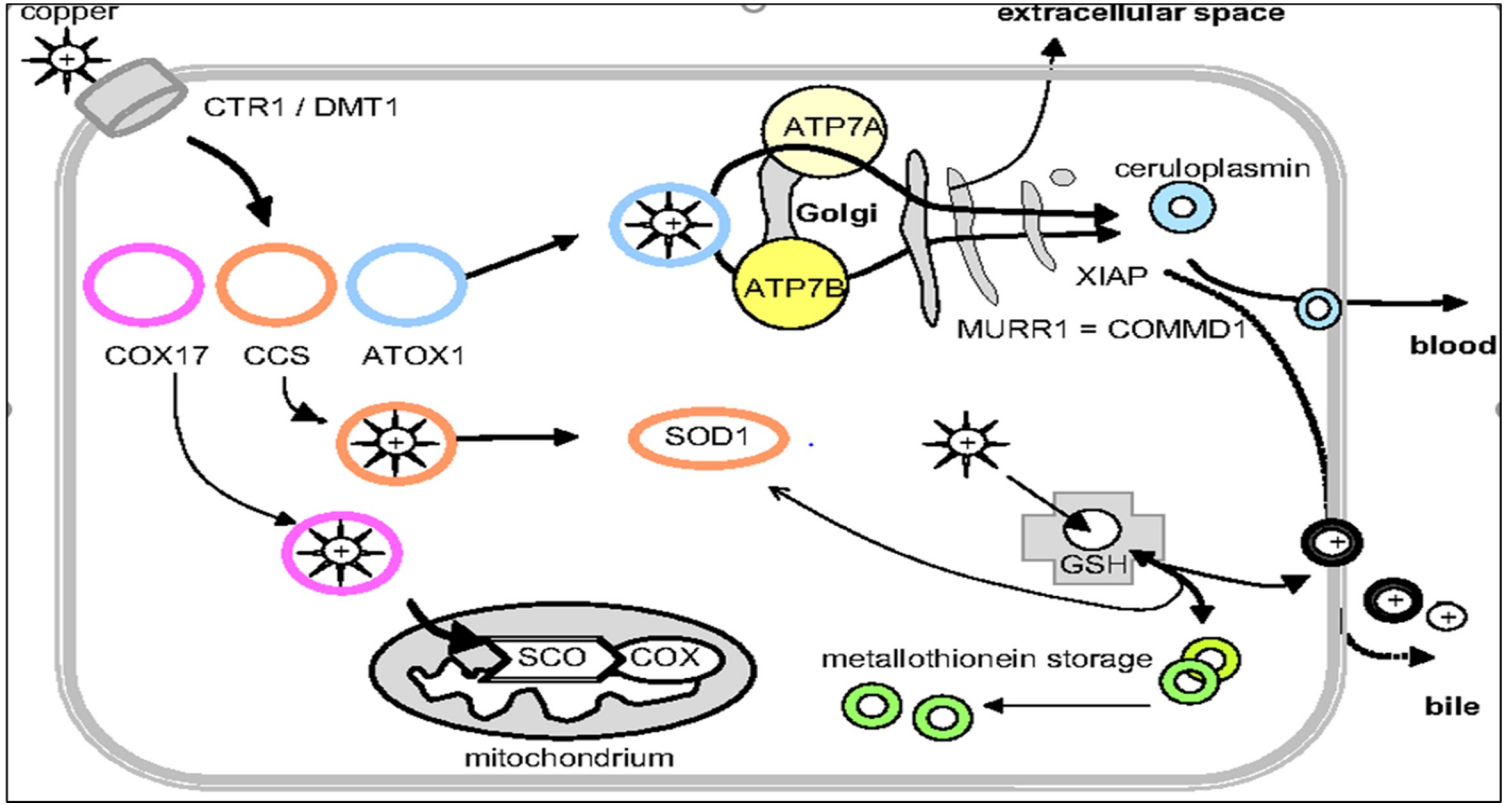



**Figure 5:** Atomic absorption spectrometry. Undifferentiated (left) and differentiated (right) cells treated with the indicated concentrations of copper for 48 h or rotenone for 24 h were analyzed by atomic absorption spectrometry to determine the intracellular copper content (**a**–**c**) and the residual content of the medium (**b**–**d**) respectively. Histogram bars represent means ± SD of three independent experiments. Values of intracellular copper were normalized on total protein and compared with untreated cells (CTR) by one-way ANOVA following Tukey test ** $p < 0.0021$ *** $p < 0.0002$

# Copper transport inside a cell




Hoffmann G. Copper-associated liver diseases. Vet Clin North Am Small Anim Pract. 2009;39(3):489-511. doi:10.1016/j.cvsm.2009.02.001

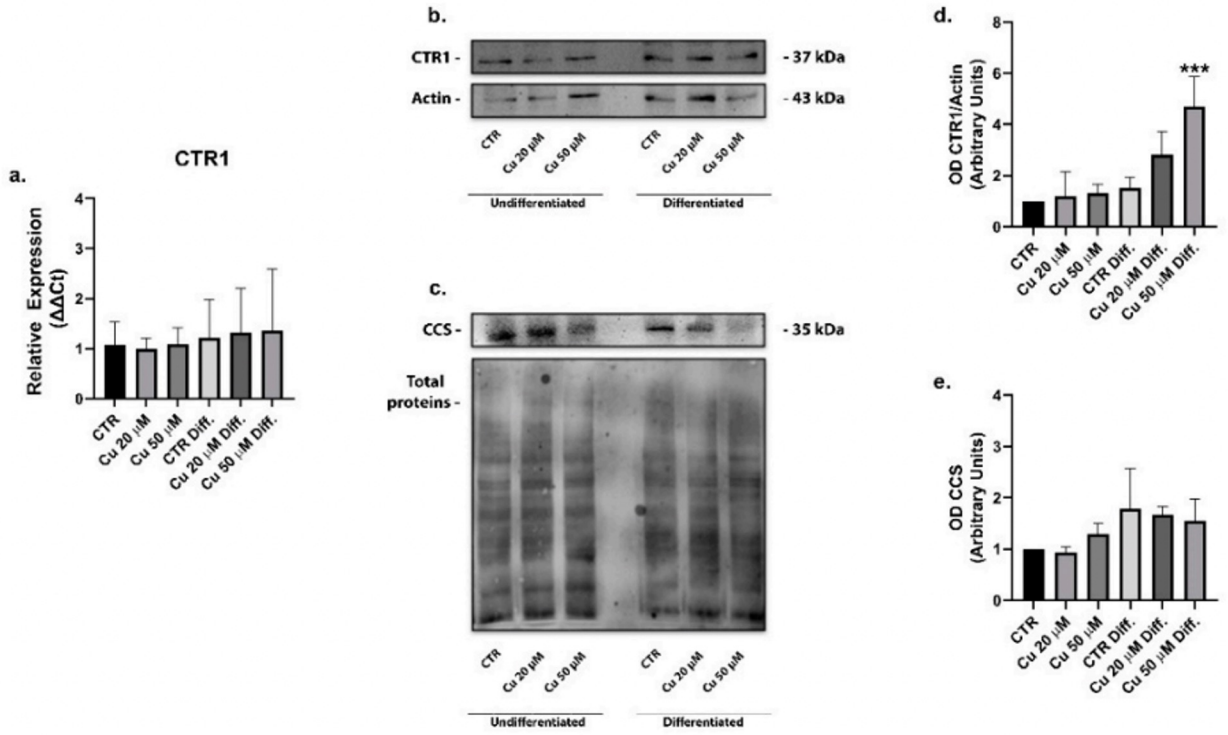



**Figure 6:** Copper treatment increased copper transporter protein 1 (CTR1) expression in differentiated SH-SY5Y cells. mRNA relative expression of the transporter CTR1 in undifferentiated and differentiated cells incubated with 20 or 50 μM copper (**a**). Representative immunoblots of the CTR1 (**b**) and copper chaperone for superoxide dismutase (CCS) (**c**) protein levels and densitometric analysis of bands representing means ± SD, respectively, of five (**d**) and three (**e**) independent experiments. β-actin was used as loading control for CTR1 (**b**), CCS levels were normalized on total protein (c). Values were compared with untreated cells (CTR) by one-way ANOVA following Tukey test *** $p < 0.0002$.

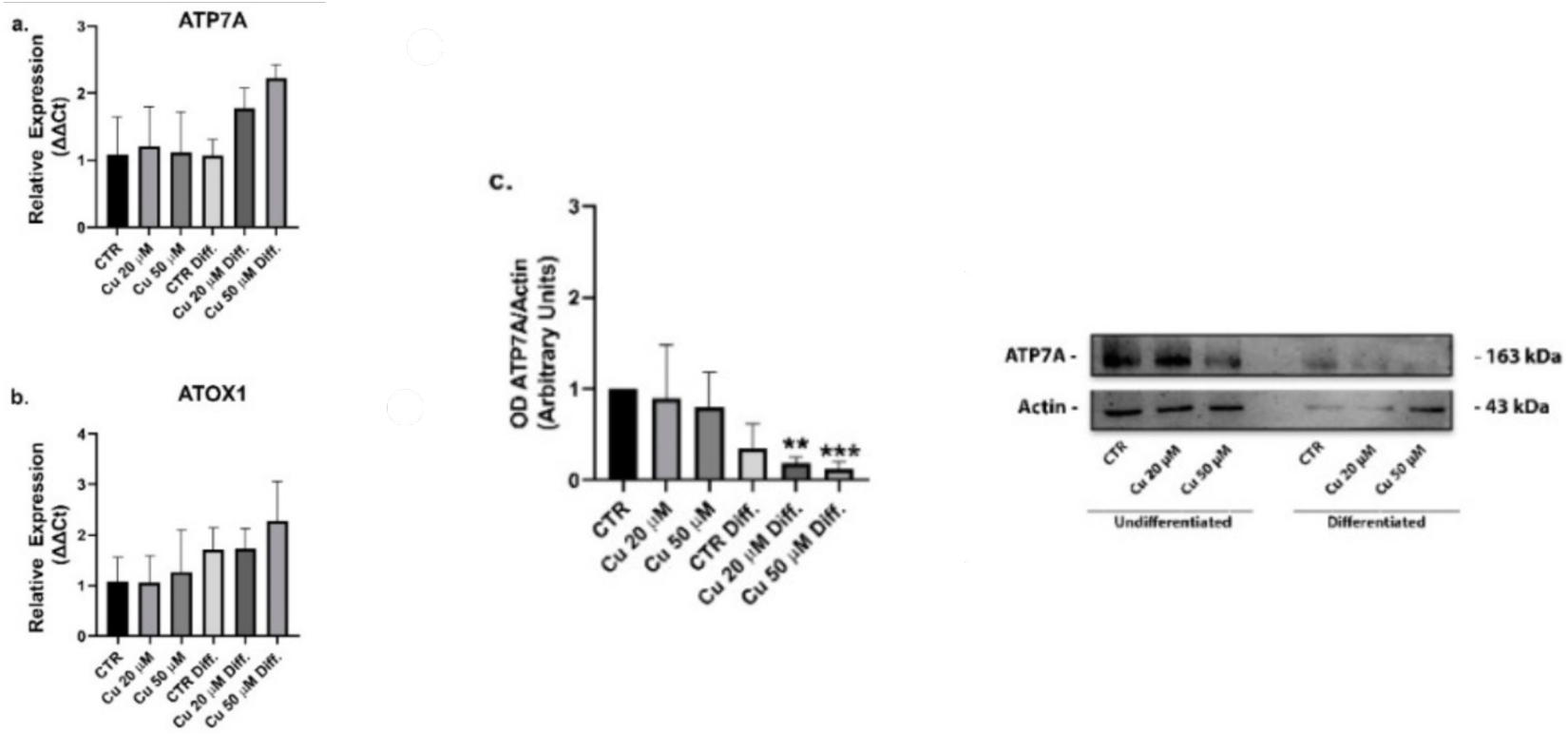



**Figure 7:** Copper treatment did not change the expression of the chaperone Atox1 and ATP7A transporter. mRNA relative expression of the transporter ATP7A (**a**) and Atox1 chaperone (**b**) in undifferentiated and differentiated cells incubated with 20 or 50 μM copper. Representative immunoblots of the ATP7A protein and densitometric analysis of bands representing means ± SD of three independent experiments (**c**). β-actin was used as loading control. Values were compared with untreated cells (CTR) by one-way ANOVA following Tukey test ** $p < 0.0021$ *** $p < 0.0002$.

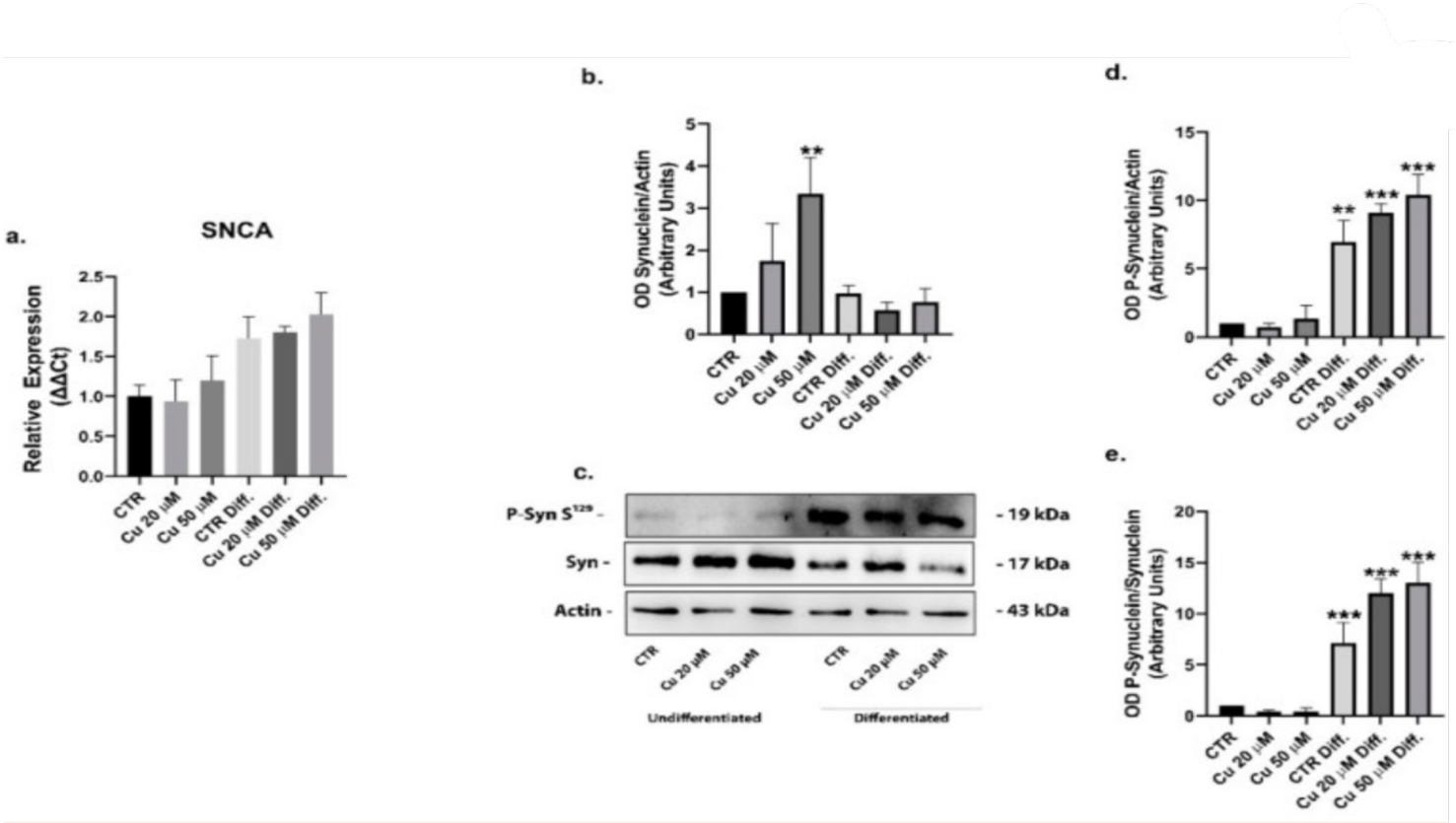



**Figure 8:** Copper treatment increased phospho-α-synuclein levels in differentiated SH-SY5Y cells. mRNA relative expression of α-synuclein gene (SNCA) in undifferentiated and differentiated cells incubated with 20 or 50 µM copper (a). Representative immunoblots of the α-synuclein and phospho-α-synuclein protein (c) and densitometric analysis of bands representing means ± SD of three independent experiments (b,d,e). β-actin was used as loading control. Values were compared with untreated cells (CTR) by one-way ANOVA following Tukey test ** $p < 0.0021$ *** $p < 0.0002$

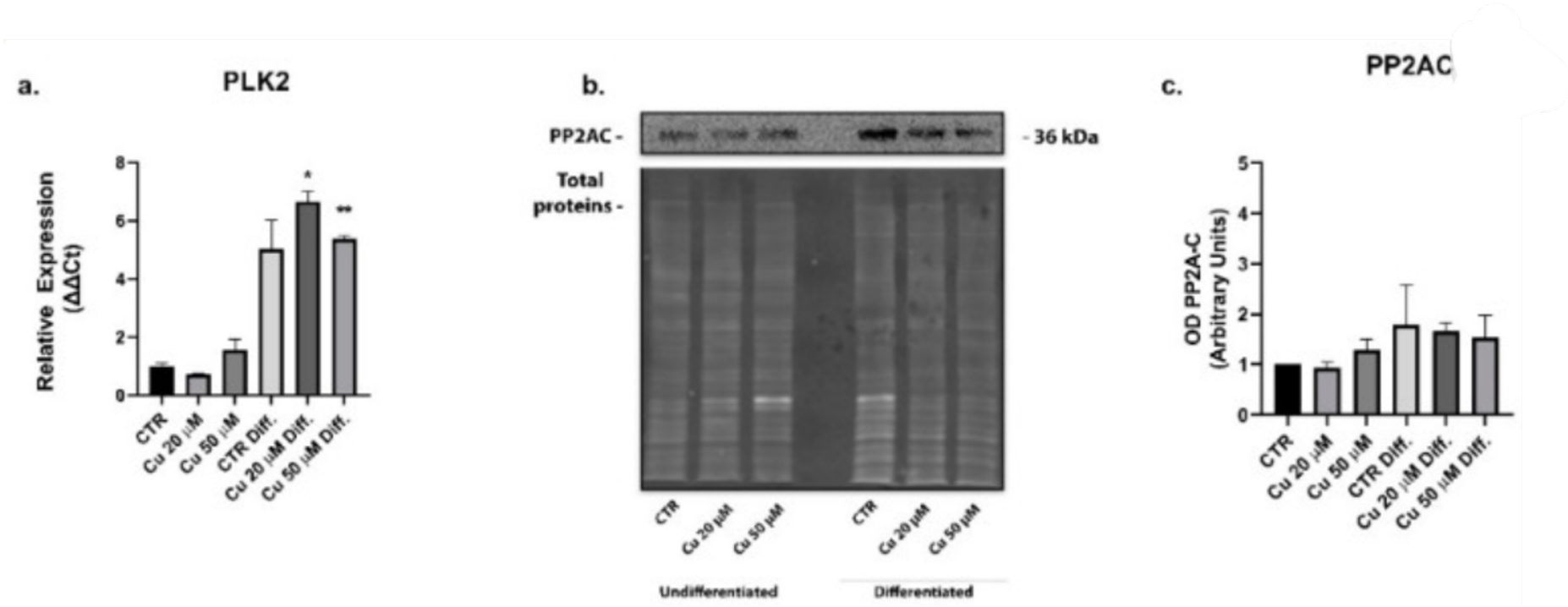



**Figure 9:** Copper treatment induced the expression of the kinase PLK2 in differentiated SH-SY5Y cells. mRNA relative expression of PLK2 in undifferentiated and differentiated cells incubated with 20 or 50 μM copper (**a**). Representative immunoblots of the phosphatase PP2A (**b**) and densitometric analysis of bands representing means ± SD of three independent experiments (**c**). PP2A values were normalized on total proteins. Values were compared with untreated cells (CTR) by one-way ANOVA following Tukey test * $p < 0.0332$ ** $p < 0.0021$

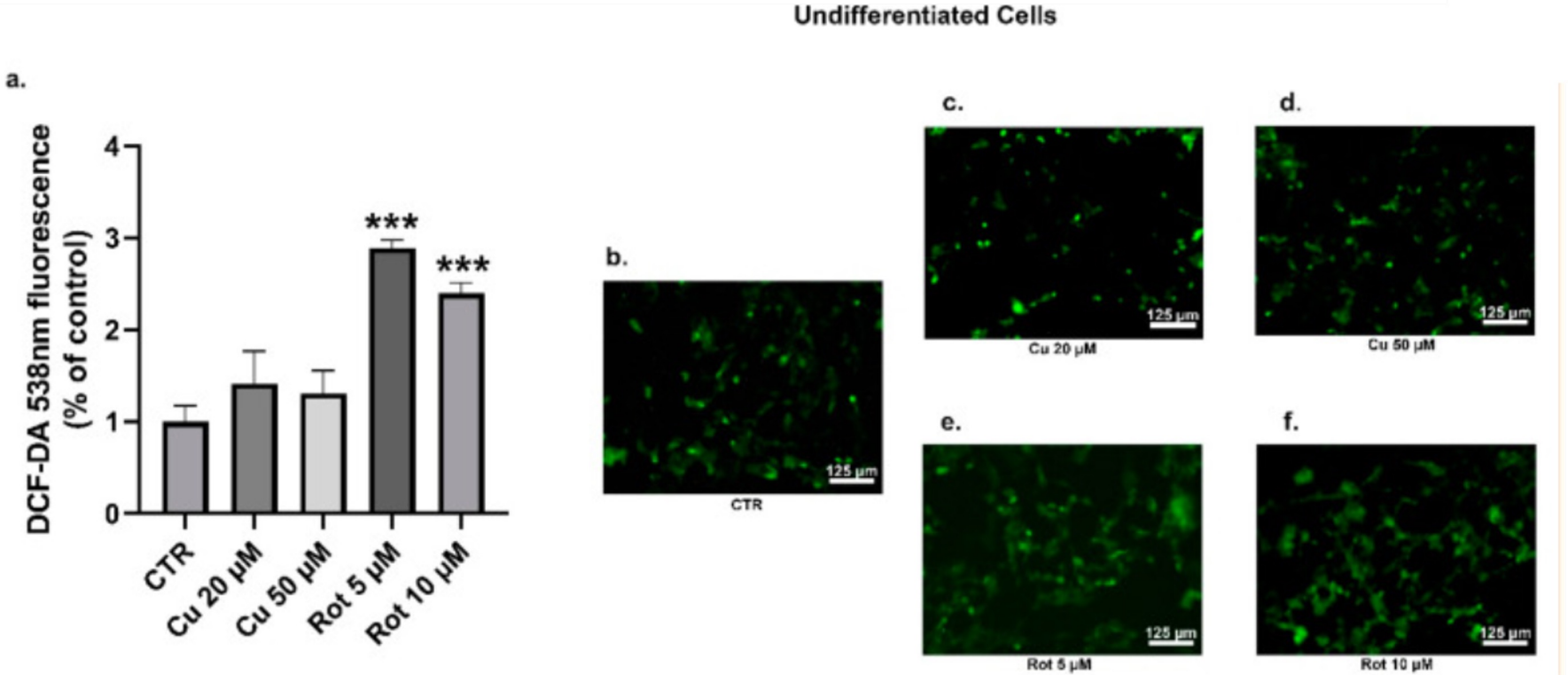



**Figure 10:** Undifferentiated SH-SY5Y cells DCFDA analysis. The relative intensity of DCF was employed to evaluate the reactive oxygen species (ROS) levels. Cells were treated with 20 or 50 µM copper (**c**,**d**) or 5 or 10 µM rotenone (**e**,**f**) and compared to untreated cells (**b**). Fluorescence values were normalized on total protein (**a**). Values were compared with untreated cells (CTR) by one-way ANOVA following Tukey test *** $p < 0.0002$

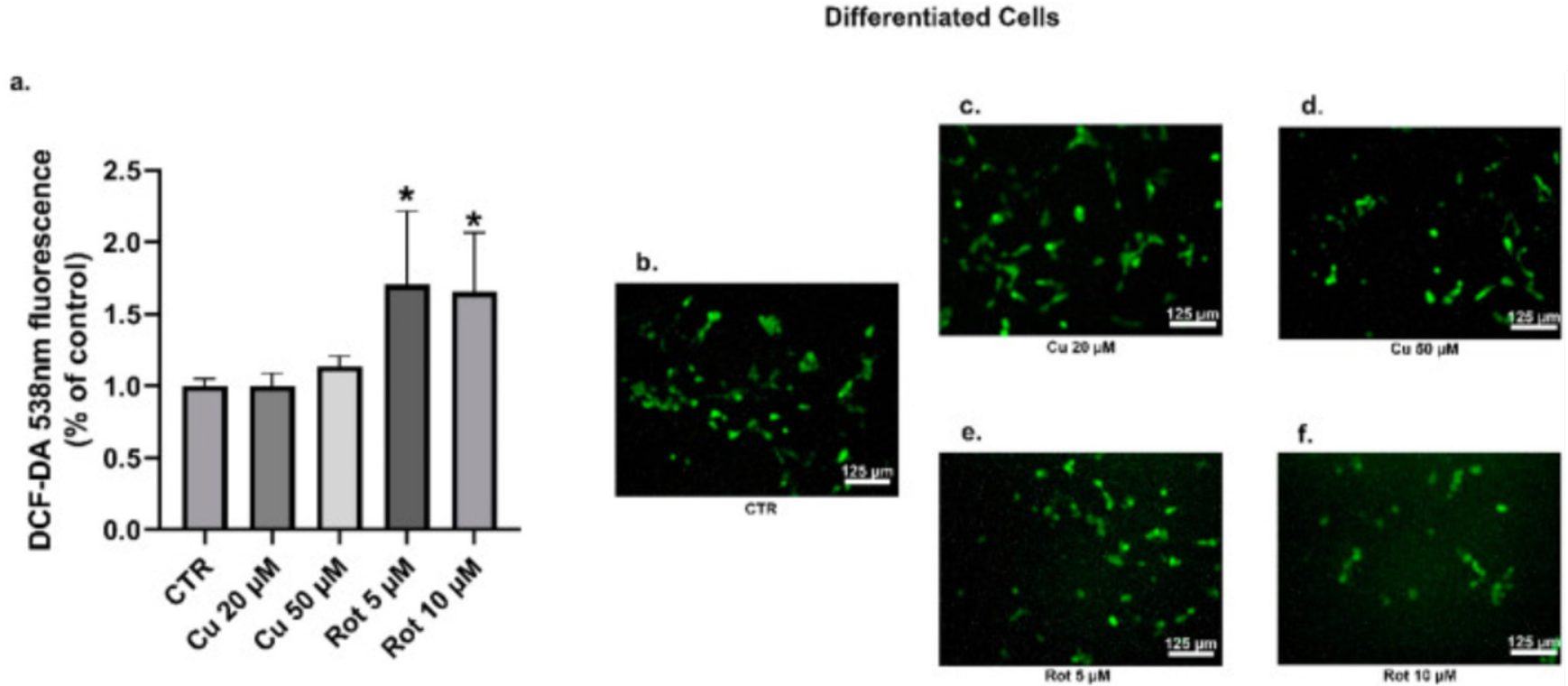



**Figure 11:** Differentiated SH-SY5Y cells DCFDA analysis. The relative intensity of DCF was employed to evaluate the ROS levels. Cells were treated with 20 or 50 µM copper (**c**,**d**) or 5 or 10 µM rotenone (**e**,**f**) and compared to untreated cells (**b**). Fluorescence values were normalized on total protein (**a**). Fluorescence values were normalized on total protein (a). Values were compared with untreated cells (CTR) by one-way ANOVA following Tukey test * $p < 0.0332$.

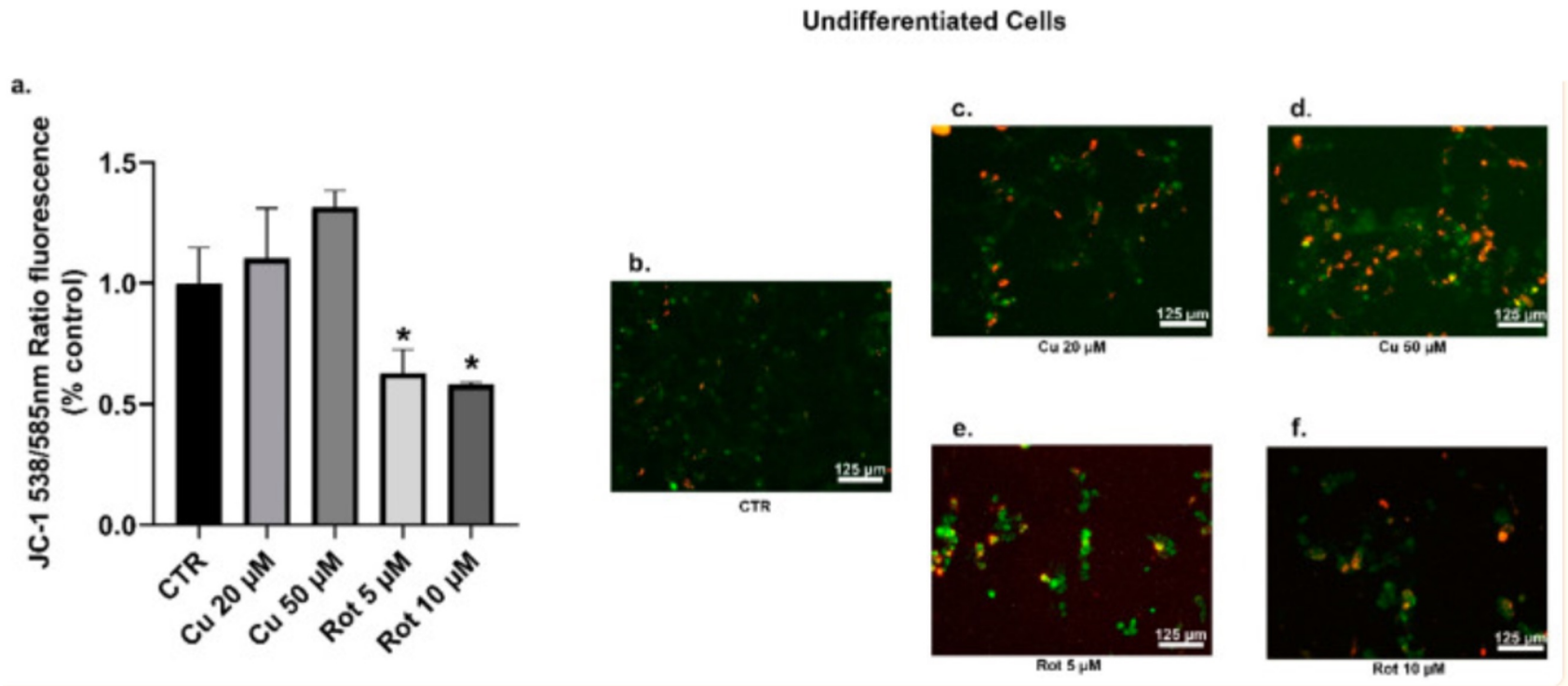



**Figure 12:** Undifferentiated SH-SY5Y cells JC-1 analysis. The JC-1 staining was used to assess the mitochondrial membrane potential (MMP) cells were treated with 20 or 50 µM copper (**c**,**d**) or 5 or 10 µM rotenone (**e**,**f**) and compared to untreated cells (**b**). The percentages of JC-1 monomers presented the percentages of SH-SY5Y cells with low MMP. Fluorescence values were normalized on total protein (**a**). Values were compared with untreated cells (CTR) by one-way ANOVA following Tukey test * $p < 0.0332$

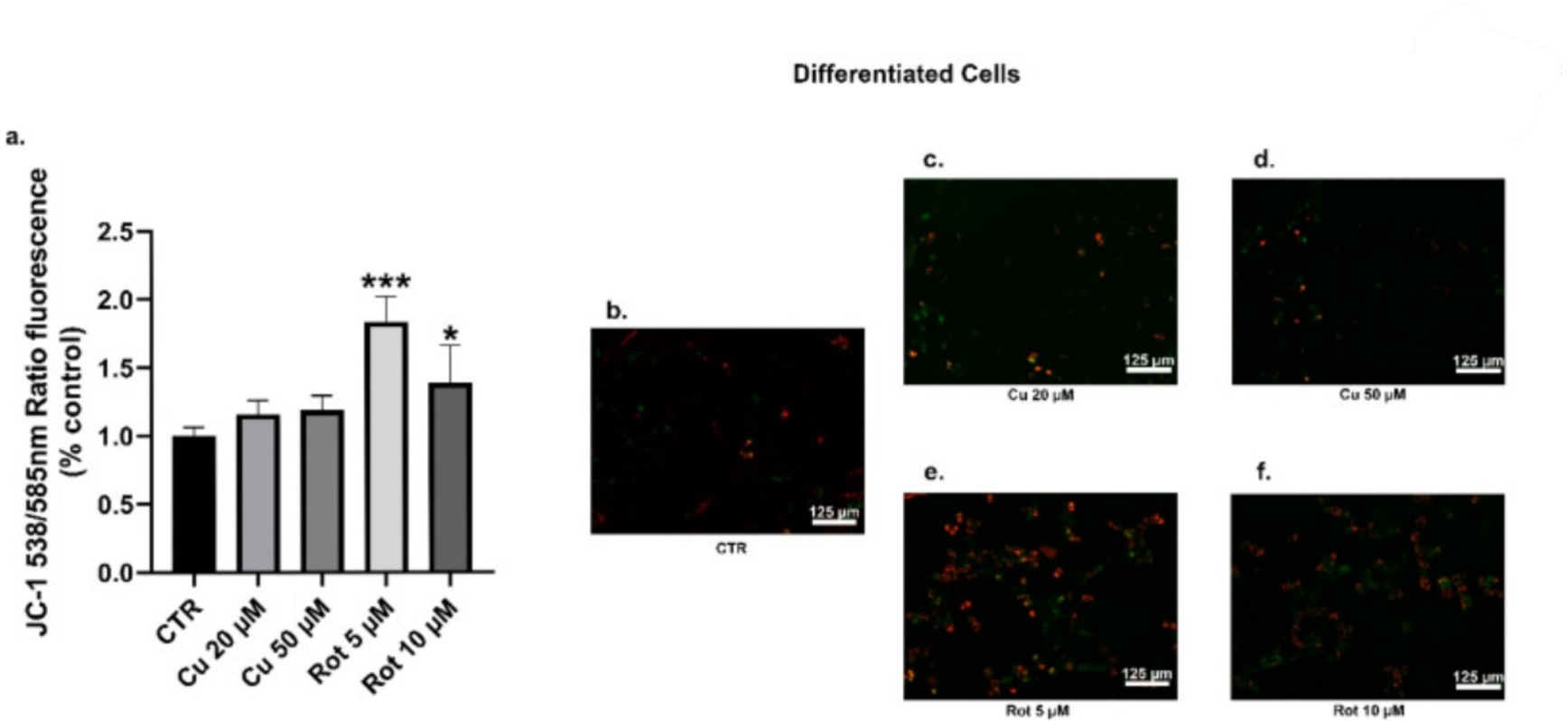


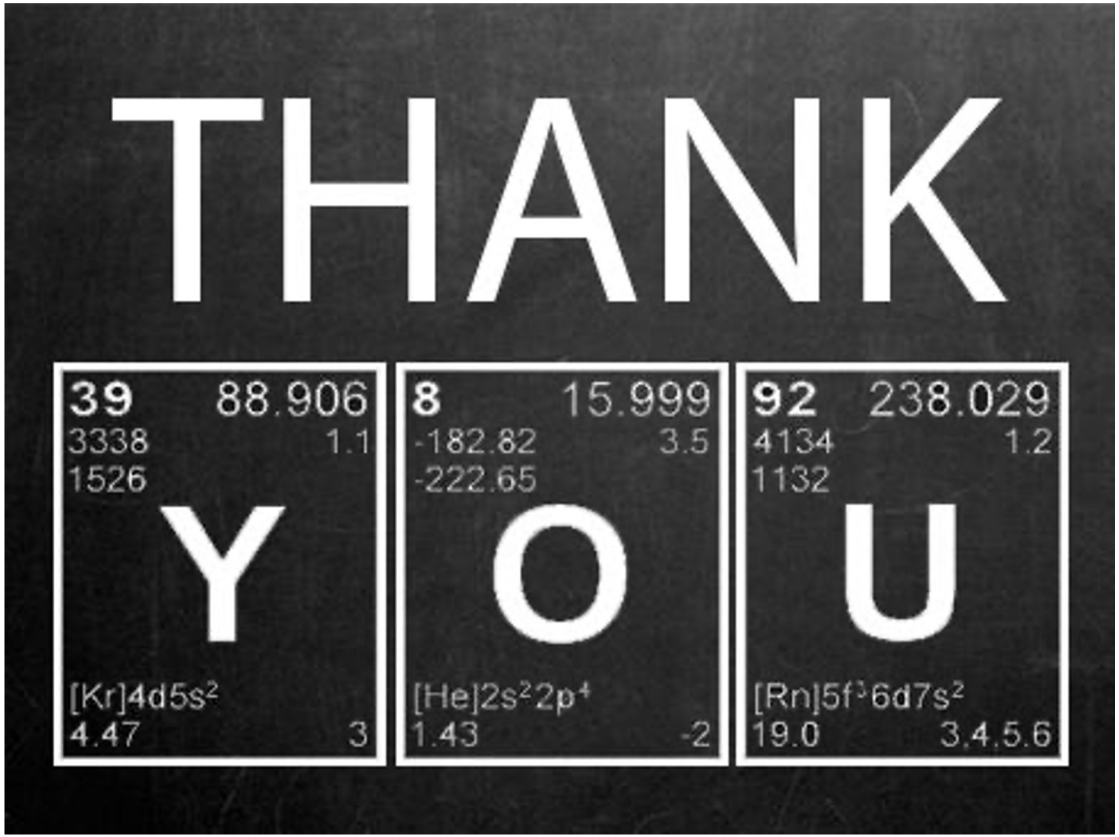

**Figure 13:** Differentiated SH-SY5Y cells JC-1 analysis. The JC-1 staining was used to assess the MMP. Cells were treated with 20 or 50 µM copper (**c**,**d**) or 5 or 10 µM rotenone (**e**,**f**) and compared to untreated cells (**b**). The percentages of JC-1 monomers presented the percentages of SH-SY5Y cells with low MMP. Fluorescence values were normalized on total protein (**a**). Values were compared with untreated cells (CTR) by one-way ANOVA following Tukey test * $p < 0.0332$ *** $p < 0.0002$.

# Conclusion

- Neuronal-like differentiated cells to copper treatment for 48 h caused an accumulation of the phosphorylated α-synuclein, similar to what happens during the pathogenesis of the PD.
- These findings advance the understanding of the correlation among copper and the formation of inclusion in PD.

## Limitations/future studies

- No rotenone group was included when looking at the α-synuclein and PLK2 enzyme levels. It will be useful to compare the copper treated cells group with the rotenone treated cells group to understand the level of toxicity.
- No explanation was provided with regards to low levels of ATP7A levels observed in copper treated differentiated cells group.
- Since copper is involved in ATP production, the concentrations tested will not have impact on mitochondrial membrane permeability and oxidative stress. Higher concentrations of copper should be tested
- To say no oxidative stress was observed, other biomarkers such as copper excretory protein ATP7B and copper binding ceruloplasmin levels should be tested. Cells can increase these markers to combat increased copper levels.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