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Christopher Gadomski April 10, 2023

Analyzing the localization of LSD1 in Tau transfected HEK293 cells: a novel method for exploring biological mechanisms of tauopathy and potential therapeutics

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Abstract

Analyzing the localization of LSD1 in Tau transfected HEK293 cells: a novel method for exploring biological mechanisms of tauopathy and potential therapeutics

By Christopher Gadomski

Background: Alzheimer's Disease (AD) as a secondary tauopathy is characterized by the presence of amyloid beta plaques and neurofibrillary tangles of phosphorylated tau. However, despite decades of research efforts, there is a lack of significant clinical treatments of AD and other tauopathies. Further research is required to explore the complexities of these diseases and create treatments that target the etiological mechanisms of these pathologies. In this study, we present a brief review of the evidence for the role of LSD1 in tau mediated neurodegeneration and demonstrate further evidence of an interaction between LSD1 and pathological tau: a novel method for future drug discovery pursuits.

Methods: HEK 293 cells were transfected with tau and stained for LSD1. Subsequently, CRISPR-modified HEK293 cells with endogenous Green Fluorescent Protein (eGFP)-tagged LSD1 were transfected with various tau mutations, and DNA was stained with NucRed 647. Cell cycle was synchronized via thymidine block, and cells were imaged periodically to determine LSD1 localization in relation to tau. HEK cells were transfected with tau and LSD1 and proteins were analyzed by western blot.

Results: Tau transfection is incredibly cytotoxic to cells and causes LSD1 mislocalization disrupting normal cell cycle. Despite the colocalization of LSD1 and tau, they fail to appear together in pulldown experiments.

Conclusion: These findings demonstrate that tau interferes with the nuclear localization of LSD1 in HEK cells. This observation provides further support that tau prevents LSD1 nuclear localization by blocking the nuclear localization signal (NLS) and can be tested in this system in future experiments. These results offer a novel target for the treatment of AD and other tauopathies. These protocols are promising first steps for drug discovery processes aimed to prevent LSD1-mediated neurodegeneration in AD and other tauopathies.

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Chapter I

Introduction

1.1 Despite increasing prevalence Alzheimer's Disease lacks effective treatment

Alzheimer's Disease (AD) is the most common form of dementia in the US, affecting cognition, memory, and behavior, and leading to a drastic decrease in the quality of life of patients and their families. Approximately 5.7 million patients live within the US alone, and projections estimate AD prevalence will increase 242% to 13.8 million by 2050 (Alzheimer's Association 2018). The CDC reports AD as the 7th leading cause of death (Murphy et al 2021). Notably, while the death rates of other leading causes of death such as heart disease, stroke and prostate cancer have decreased, deaths due to AD have increased 123% from 2000 to 2015 (Alzheimer's Association 2018). In addition, AD costs around $305 billion dollars annually in the US and places a heavy emotional burden on family and caretakers (Wong 2020).

Neurodegeneration that underlies AD and other dementias is complex and multifactorial and is characterized by the progressive death of neurons and subsequent loss of neuronal connectivity. While current treatments target specific biomarkers of AD, they do not alter disease progression. Further research is crucial in finding a treatment that slows the progression of the disease or prevents it entirely (Karlawish, Grill 2021). AD is characterized by the aggregation of extracellular amyloid-beta (Aβ) plaques and extracellular and intraneuronal tau neurofibrillary tangles (NFTs). Recently both *in vitro* and *in vivo* studies have demonstrated that tau is required for Aβ- induced neurotoxicity. In AD, hyperphosphorylated tau NFTs highly correlate with the level of cognitive impairment (Benjanin et al. 2017, Wand and Mandelkow, 2016). However exact mechanisms underlying tau-mediated neurodegeneration remain unclear.

1.2: Significance of tau in neurodegeneration

Tau is an intrinsically disordered protein that has been well characterized as a Microtubule-associated Protein (MAP) that canonically helps promote microtubule assembly and stabilization. Tau has also been shown to have additional roles in cellular processes such as neurogenesis, neuronal activity, iron export, and long-term depression (Hernandez and Avila, 2007, Wang and Mandelkow, 2016). While tau shows little tendency for aggregation in its native unfolded state, aberrant isoforms and excess amounts of tau are known to have pathological effects (Avila 2010).

Tauopathies are neurodegenerative diseases characterized by the presence of tau aggregates and/or phosphorylation. Although the exact mechanisms underlying tau pathology are contested, tau aberrant metabolism leads to structural changes, hyperphosphorylation and aggregation, (Avila, 2010, Hernandez and Avila 2007, Wang and Mandelkow, 2016). While AD is the most prevalent secondary tauopathy, primary tauopathies include diseases such as corticobasal degeneration, Pick's Disease, frontotemporal lobar degeneration, and progressive supranuclear palsy, which are associated with distinct tau isoforms resulting from alternative splicing of exons 2, 3, and 10.

There is strong evidence that pathological tau mediates neurotoxic effects (e.g., synaptic loss, genetic expression changes, increased inflammatory cascade, and cell death) leading to neurodegeneration in these tauopathies (Roberson et al., 2007, Wang and Mandelkow, 2016). Additionally, it is believed that pathological tau may propagate in a prion-like manner. Tau aggregates have been shown to seed further tau aggregation in nearby cells as intracellular tau is cleared and becomes extracellular and then seeds more aggregations in nearby cells (Strang et

al., 2018). Given the rising incidence of AD and other tauopathies, mechanistic studies targeting pathological tau and its downstream effects are crucial to further our understanding of AD and develop novel therapeutic strategies.

1.3: Lysine-specific demethylase 1 is a potential therapeutic target of AD/tauopathies

Lysine-specific demethylase 1 (LSD1/KDM1A) is a nuclear protein that regulates histone methylation by removing mono- and di-methyl groups on lysine 4 of histone 3 (H3K4). Histone methylation plays an important role in cell fate in keeping track of past transcription. Therefore, failure of LSD1 to continually maintain histone methylation in differentiated cells may play a significant role in disease (Lee and Katz, 2020). Additionally, a neuronal specific splicing variant of LSD1 plays an important role in neurite maturation and morphology. LSD1 knockdown and overexpression causing neurite maturation inhibition and enhancement. Tau neuroinflammatory pathology reduces neurite density in AD (Sone et al., 2020), these evidences when taken together further suggest the role of LSD1 in the pathogenesis of many neurodegenerative disorders (Zibetti et al., 2010).

Work previously done by the Katz lab demonstrates that tau may inhibit proper LSD1 functioning by sequestering LSD1 and preventing proper nuclear enrichment via evidence of LSD1 mislocalization to tau in human AD cases and PS19 mice (Christopher et al. 2017, Engstrom et al., 2020). Inducible LSD1 depletion in adult mice leads to learning deficits, memory deficits, and significant neuronal cell death in the cortex and hippocampus (Christopher et al. 2017). LSD1 deletion results in transcriptional alterations that significantly overlap with gene expression changes in postmortem human AD and frontotemporal dementia cases but not other neurodegenerative diseases, which underscores the importance of proper LSD1 functioning

(Christopher et al. 2017). To further explore the functional interaction between LSD1 and tau we tested the impact of LSD1 reduction and overexpression in tau PS19 mice, where mice express a P301S mutation of tau that leads to tau protein hyperphosphorylation and aggregation. LSD1 reduction exacerbates tauopathy phenotypes (neurodegeneration, transcription alterations) and subsequent LSD1 overexpression temporarily suppresses neuronal cell death even in the presence of pathological tau (Engstrom 2020). The combination of these data from both human cases and mouse models suggests pathological tau sequesters LSD1 in the cytoplasm, leading to neuronal cell death. To prevent the mislocalization of LSD1 and LSD1's loss of function, it will be interesting to study the potential interaction between LSD1 and pathological tau.

Despite the evidence demonstrating LSD1-tau colocalization we have not been able to pull down both proteins together, suggesting they are not binding directly. Because tau is an intrinsically disordered protein with prion-like seeding and LSD1 also has an intrinsically disordered domain at the N terminus, it is possible that these charged regions are weakly associated with each other when LSD1 and tau colocalize. Furthermore, It is well established that the nuclear localization of LSD1 relies on the nuclear localization signal (NLS) which is located within LSD1's intrinsically disordered domain (Jin et al. 2014). We hypothesize that pathological tau sequesters LSD1 by interacting with the disordered domain of LSD1 thereby blocking the NLS and preventing nuclear entry. We propose that this interaction can be targeted therapeutically in AD and other tauopathies. This thesis details *in vitro* experiments that aim to characterize the interaction between LSD1 and tau. This methodology can be used to screen for a drug to block tau-mediated LSD1 loss of function and further explore mechanisms underlying the LSD1-tau interaction.

Chapter II

Methodology

To explore the LSD1 tau interaction, we have used a combination of live cell imaging and immunohistochemistry of fixed cells. For live imaging experiments a HEK 293 CRISPR eGFP tagged LSD1 cell line (ThermoFisher Scientific) was used to visualize LSD1 throughout the cell cycle.

2.1: Protocols to explore LSD1-tau interaction *in vitro*

2.1A: Cell Culture

HEK293 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium 11965092) supplemented with 10% Gibco heat inactivated Fetal Bovine serum (16140063) and Penicillin-Streptomycin-Glutamine 100X (10378016) at 37ºC and 5% CO2. Gibco Fluorobrite DMEM (A1896701) was used to minimize background in live imaging experiments. Cells were passaged to maintain linear growth rate upon reaching 60 - 80% confluency. 16 hour thymidine blocks (2mM final concentration) followed by PBS washes (3x) were used to synchronize the cell cycle by halting the progression of the cell cycle at the G1/S barrier before DNA synthesis.

2.1B: Transfection

Polyplus jetPrime DNA transfection reagent (114-07) was used to transfect plasmids according to the following procedure. Cells were seeded the day prior to transfection according to Polyplus recommendations to be 60-80% confluent at the time of transfection. After adding plasmids to the jetPrime buffer and mixing, jetPrime reagent was added. Solution was mixed, spun down and allowed to incubate for 10 minutes before being added to the cells. The jetPrime transfection relies on jetPrime forming positively charged complexes with DNA that enter the cell through endocytosis to reach the nucleus during mitosis when the nuclear envelope

dissolves. Incubation time varied depending on experiment with plasmid expression beginning at 12 hour and reaching a maximum at around 36.

2.1C: Immunostaining

When staining, special care was taken to add and remove solutions gently to minimize losing cells. After washing cells two times with PBS, cells were fixed via 20 minute wash with 4% PFA in PBS. Cells were washed 3 additional times for 5 minutes each before permeabilization via 10 minute wash in 0.25% Triton-x100 (in H2O). Three additional 5 minute PBS washed prior to 1hr blocking with 10% goat serum (in PBS). Cells were incubated overnight at 4ºC with 1º Ab (diluted in 1% goat serum/PBS). Three PBS washes subsequent to hour-long incubation with 2º Ab (Goat anti mouse Alexa-488 diluted 1:500 in 1% goat serum). After three final PBS washes, coverslips were mounted onto slides using tweezers and ProLong mounting media. Slides were allowed to air dry for 1hr before storage in a cool dry place.

Target	Manufacturer	Clone	Dilution
Tau	invitrogen	Tau 5	2ug/mL
LSD1	Abcam 17721		1:100

Table 1. Antibody info

2.2: Live imagining of fluorophores to create time lapse movies

Nikon A1R was used for live microscopy. Heated, humidified CO2 live cell culture chamber and lens warmers were used to optimum viability. NucRed Live 647 DNA dye was used to visualize the nucleus in later experiments. Experiments were programmed to take images of set points every 15-30 minutes.

2.3 GFP-Trap and Western blot

To further study the mechanistic interaction between LSD1 and tau, chromotek GFP-trap magnetic agarose kit was used to immunoprecipitate GFP-fusion proteins from cells according to manufacturer's protocols. Western blot protocols were obtained from Dr Kenneth Moberg as described in Zhao et al., 2023.

Chapter III

Results

3.1 *In vitro* experiments offer mechanistic insight and development of potential therapeutics

The ability to recapitulate LSD1 and tau colocalization *in vitro* is incredibly useful for two reasons. First, it allows for powerful and informative experimental techniques to explore basic mechanisms that would otherwise be difficult due to economic and time constraints, and second, recapitulating loss of LSD1 nuclear enrichment subsequent to tau transfection in a live imaging context can provide the basis for a drug screen that may help target tau-mediated neurodegeneration and treat AD and other tauopathies.

3.2 Time intensive experiments require continual optimization

To recapitulate LSD1-tau colocalization *in vitro,* HEK cells were fixed and immunofluorescence staining was performed. . HEK 293 cells transfected with various tau mutations were stained for LSD1, Tau, and DAPI.We found that, regardless of tau mutation, tau transfection was very cytotoxic and as a result,LSD1 lost distinct nuclear enrichment. This cytotoxicity made staining difficult due to the low adhesion of the cells. With initial success in fixed cells, we continued experiments in CRISPR modified cells to allow for live imaging following tau transfection. Many experiments did not produce clear results due to lack of uniformity in LSD1 localization in all cells. Initial experiments did not include a synchronization of the cell cycle, but I soon noticed a pattern in LSD1's loss of nuclear enrichment. LSD1 localization follows mitosis in that after the nuclear envelope dissolves, LSD1 normally becomes diffuse before localizing to the nucleus once again at the end of mitosis, but when transfected with tau, LSD1 never regains full nuclear enrichment. This window of action led us to include the thymidine block to synchronize the cells to attempt to increase uniformity of LSD1

mislocalization. Since transfection efficiency is also dependent on the cell cycle, these experiments required additional transfection optimization to find the window of action.

3.3 Summary of results

LSD1 loses nuclear enrichment when transfected with tau (Figure 1). Healthy cells maintain high levels of clearly enriched LSD1 (Figure 1A). These regions are nuclei and colocalize to DAPI staining not shown. Tau transfection causes LSD1 to lose normal nuclear enrichment. Figure 1C-L are individual frames taken from a live imaging experiment. After transfected with tau, LSD1 loses this clear enrichment and bears resemblance to a failed attempt at mitosis (Figure 1 D-L).

Figure 1 Initial LSD1 staining and live imaging shows LSD1 losing nuclear enrichment

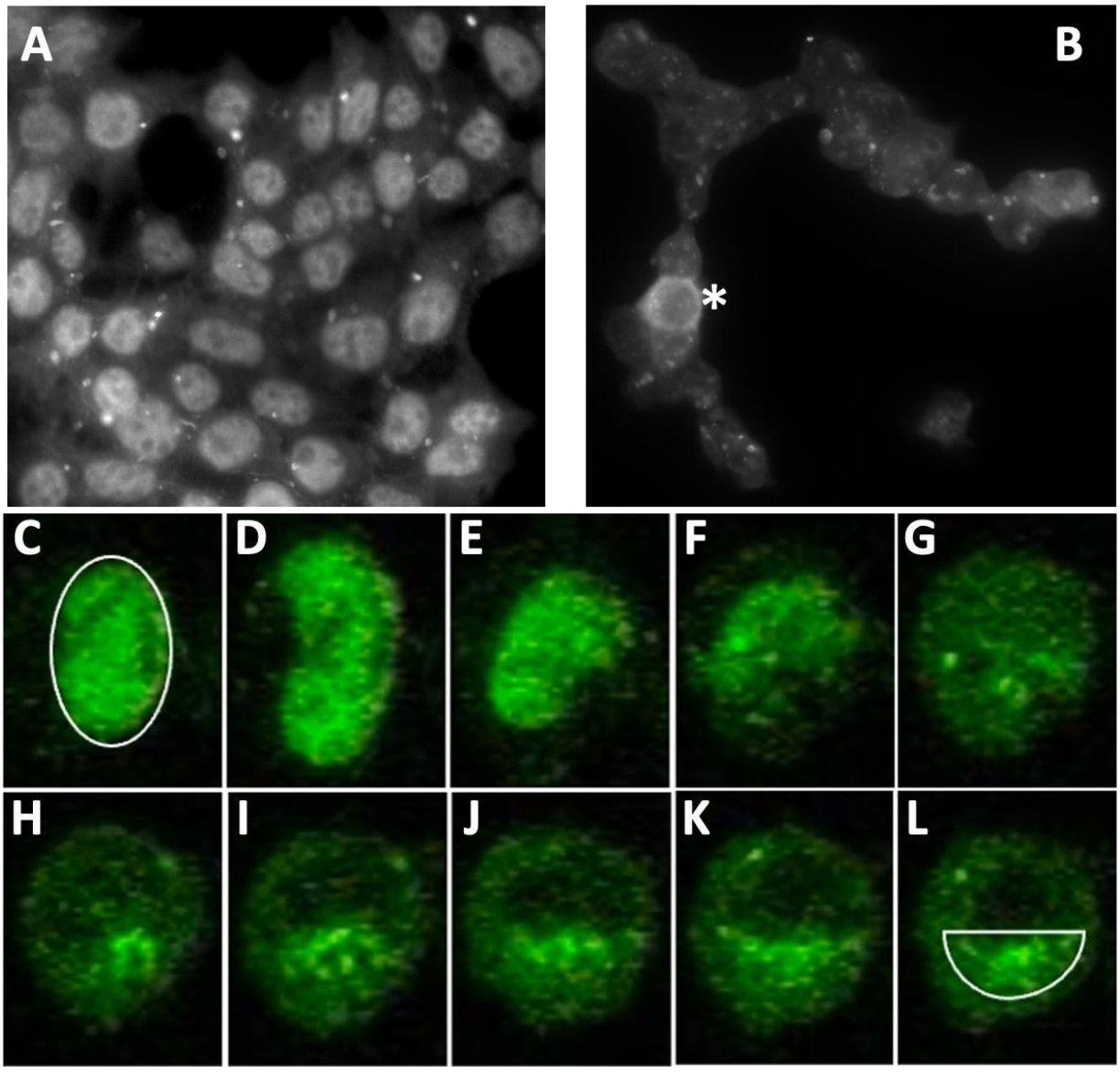


Figure 1. LSD1 is mislocalized in the presence of tau LSD1 staining demonstrates LSD1 is localized to the nucleus in control HEK293 cells **(A)**. Upon transfection with tau, LSD1 loses nuclear enrichment and becomes cytoplasmically localized (asterisk) and localized to puncta **(B).** Live imaging of eGFP tagged LSD1 HEK293 cells show LSD1 mislocalization in real time (**C-L).**

LSD1 is normally nuclear and strongly enriched in all healthy cells (Figure 2). While the transfection reagent and buffer are slightly toxic to the cells, overall the cells appear healthy and LSD1 is not diffuse but nuclear. Notably, the DNA dye has not intercalated all cells, this may be due to the dye taking longer to stain cells that are closer together or and decreased efficiency in that area over all.

Figure 2 Mock Transfection and DNA staining demonstrates LSD1 to be nuclear(Control)

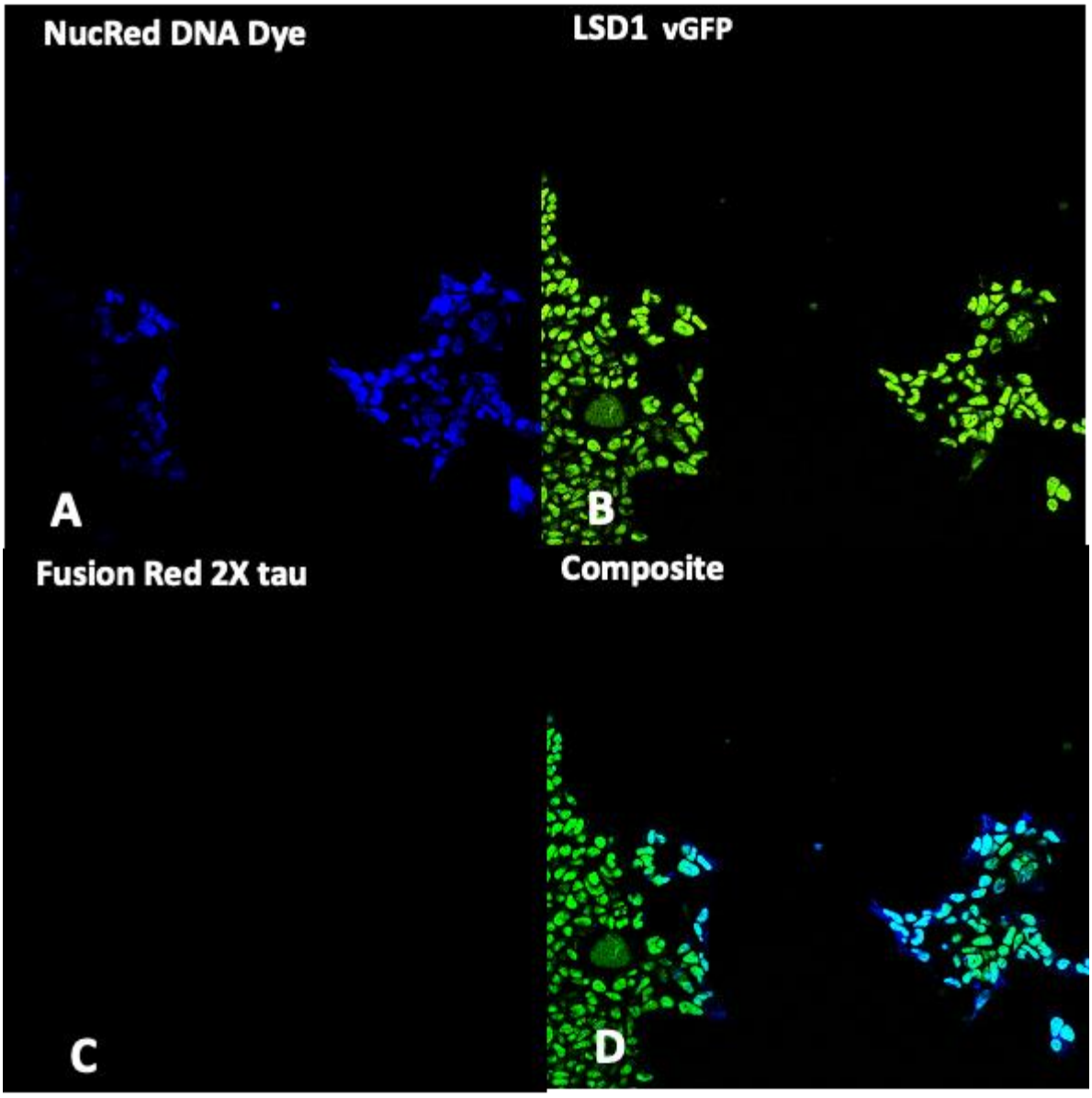
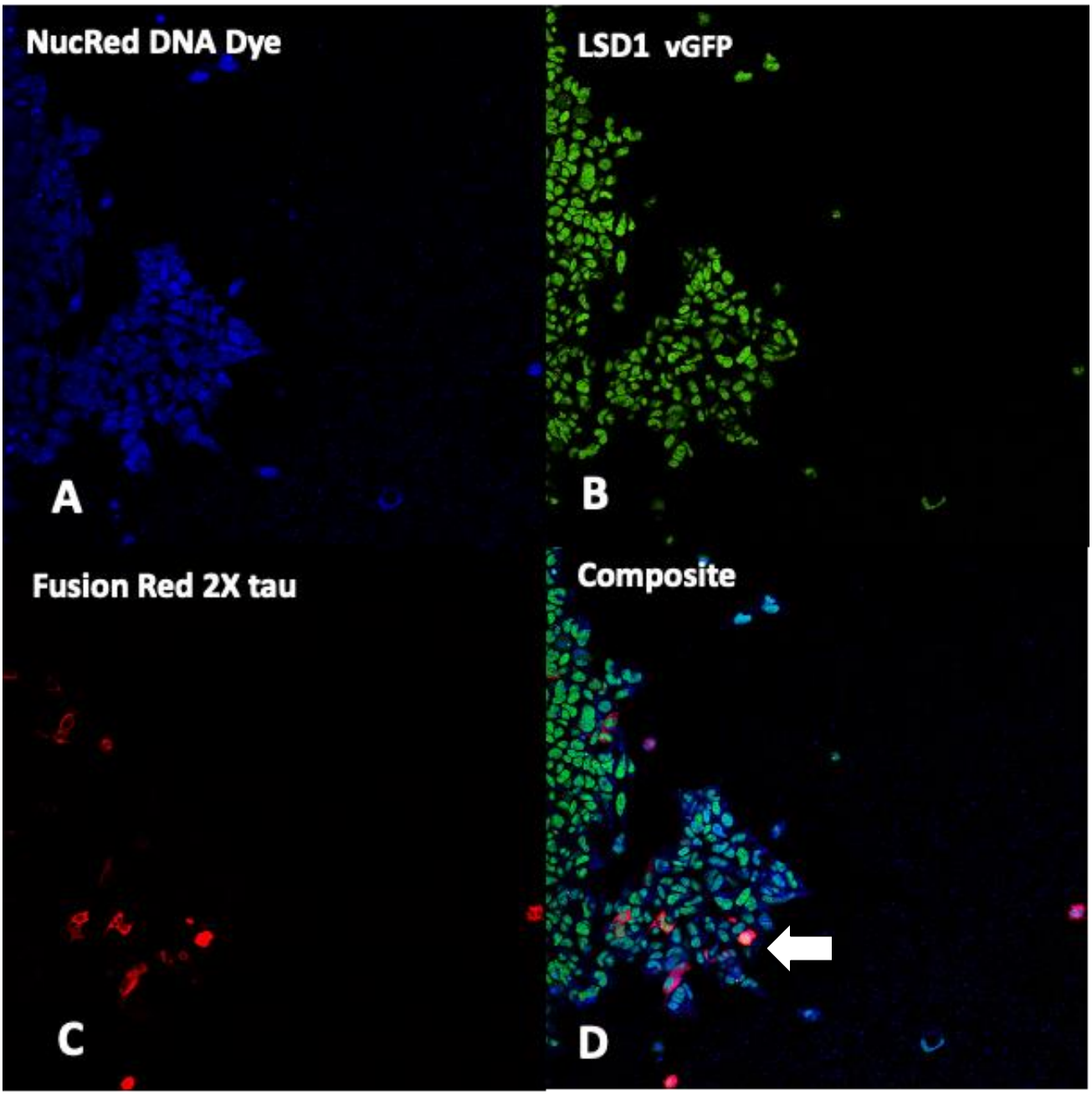


Figure 2. LSD1 is a nuclear protein. Cells treated with JetPrime transfection reagent and buffer 12 hours prior to imaging. NucRed DNA dye added 20 minutes prior to imaging. **A** NucRedDNA dye visualize clear nuclei on the right half of the image **B** LSD1 tagged with vGFP demonstrates clearly enriched healthy cells **C** Cells were treated with JetPrime reagent and

buffer but no plasmid so do not express tau as. **D** Merge image demonstrates LSD1 and DNA dye colocalization.

In these live imaging experiments there are brief moments where tau is beginning to be expressed in some cells but the cell has not yet begun to divide (Figure 3). Clear nuclear holes where tau is not expressed demonstrate that tau is cytoplasmic(Figure 3C). The arrow in Figure 3D, and Figure 3E demonstrate similar to Figure 1C the brief moment where while LSD1 is nuclear enriched but tau, DNA dye and LSD1 are all colocalized together. This suggests that the nuclear envelope has dissolved at the beginning of mitosis and Tau now has access to LSD1. It is also of note that the cells in close proximity to tau show more morphological difference than cells further away as tau has been shown to seed aggregation in nearby cells previously (Strang et al. 2018).

Figure 3 Tau transfection does not immediately colocalize to LSD1



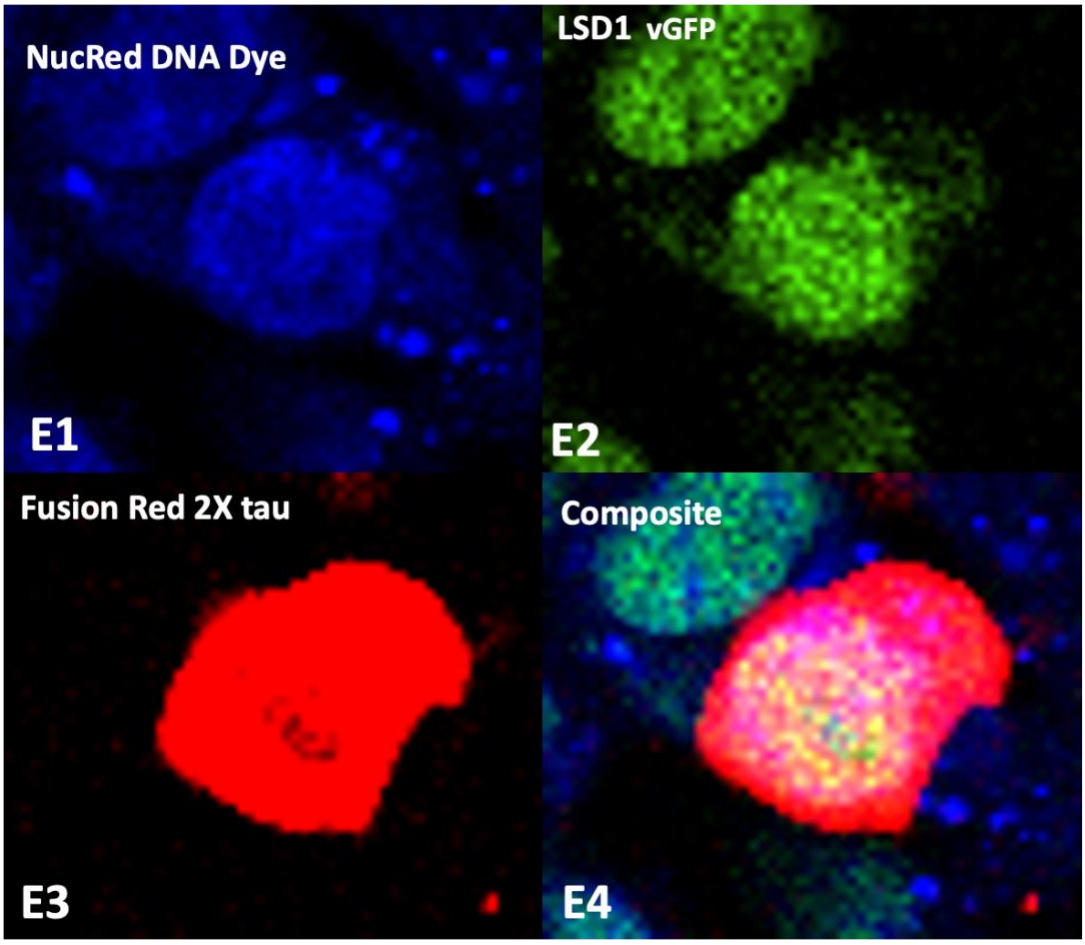
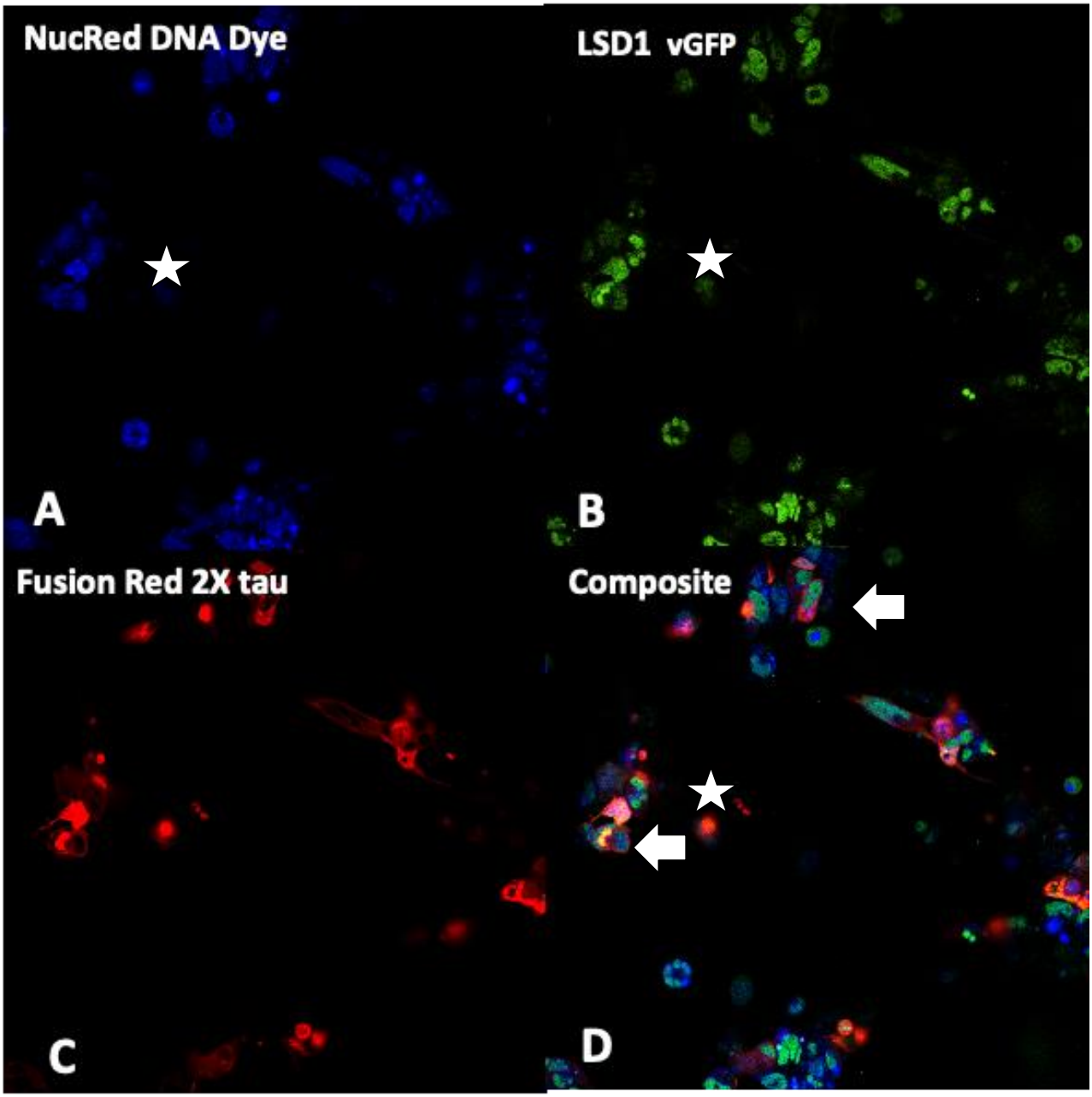


Figure 3. Cells do not not immediately lose LSD1 nuclear enrichment. Cells treated with JetPrime transfection reagent and buffer 12 hours prior to imaging. NucRed DNA dye added 20 minutes prior to imaging. **A** NucRedDNA dye visualizes clear nuclei **B** LSD1 tagged with vGFP demonstrates clearly enriched LSD1 and initial changes in morphology **C** Fusion red tagged 2X tau is not immediately expressed in all cells despite thymidine synchronization. **D** Merge image demonstrates LSD1 and DNA dye colocalization, cytoplasmic tau. **E1-4** Zoom of **A-D**

Increasing the incubation time to 36 hours leads to some cells beginning to show LSD1 localization(Figure 4E). However despite being treated with a thymidine block, the cells have fallen out of synchronization with each other and there is not a great amount of uniformity in LSD1 morphology. Interestingly, LSD1 does not localize to the DNA dye in some cells in the absence of intracellular tau expression (Figure 4D). Additionally, as previously mentioned tau is incredibly cytotoxic. As cells die they become less adherent and drift out of the focal plane, but the tau signal is always the strongest and last to lose focus. As shown by the cell beneath the star, the tau signal is incredibly strong but the LSD1 signal is very diffuse and the DNA signal is very faint compared to other cells (Figure 4). This may also be a result of insoluble tau being left behind as the cell dies.

Figure 4. LSD1 loses nuclear enrichment 21 hours after transfection



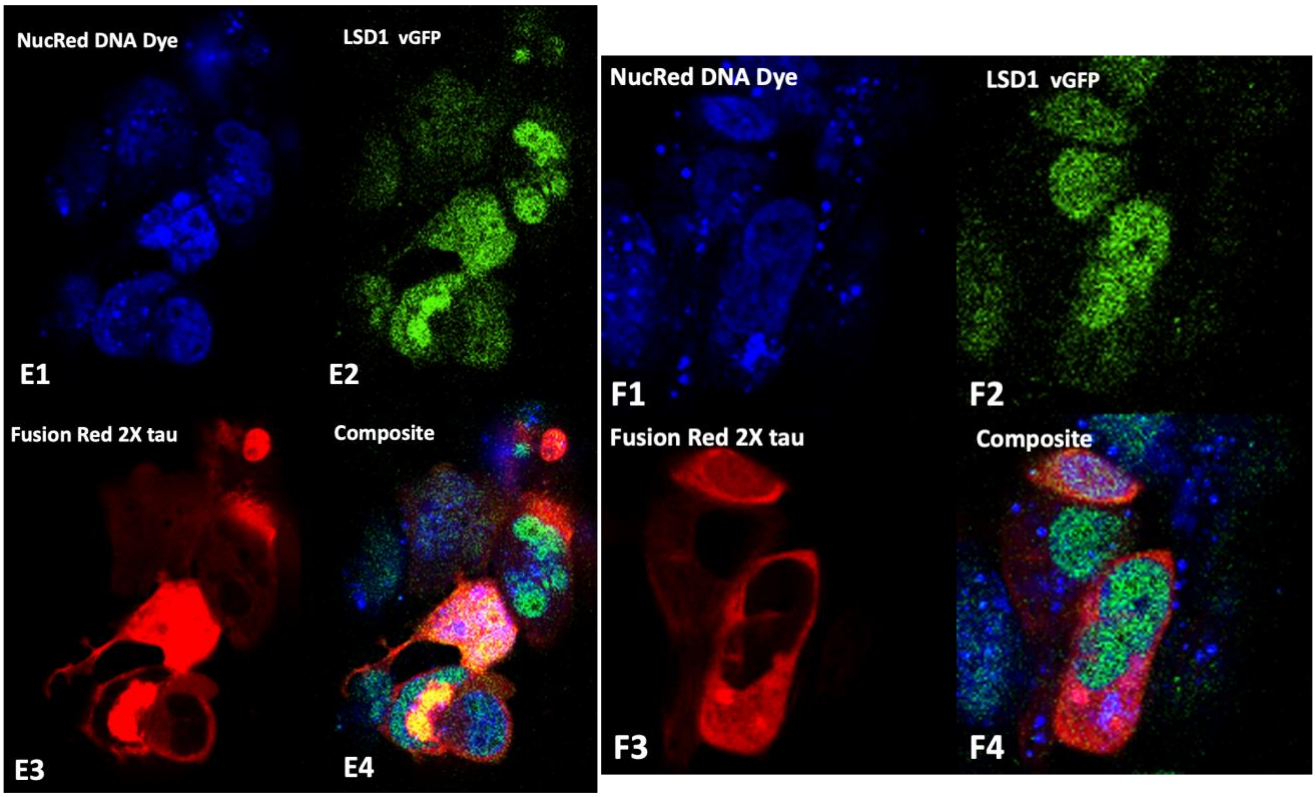
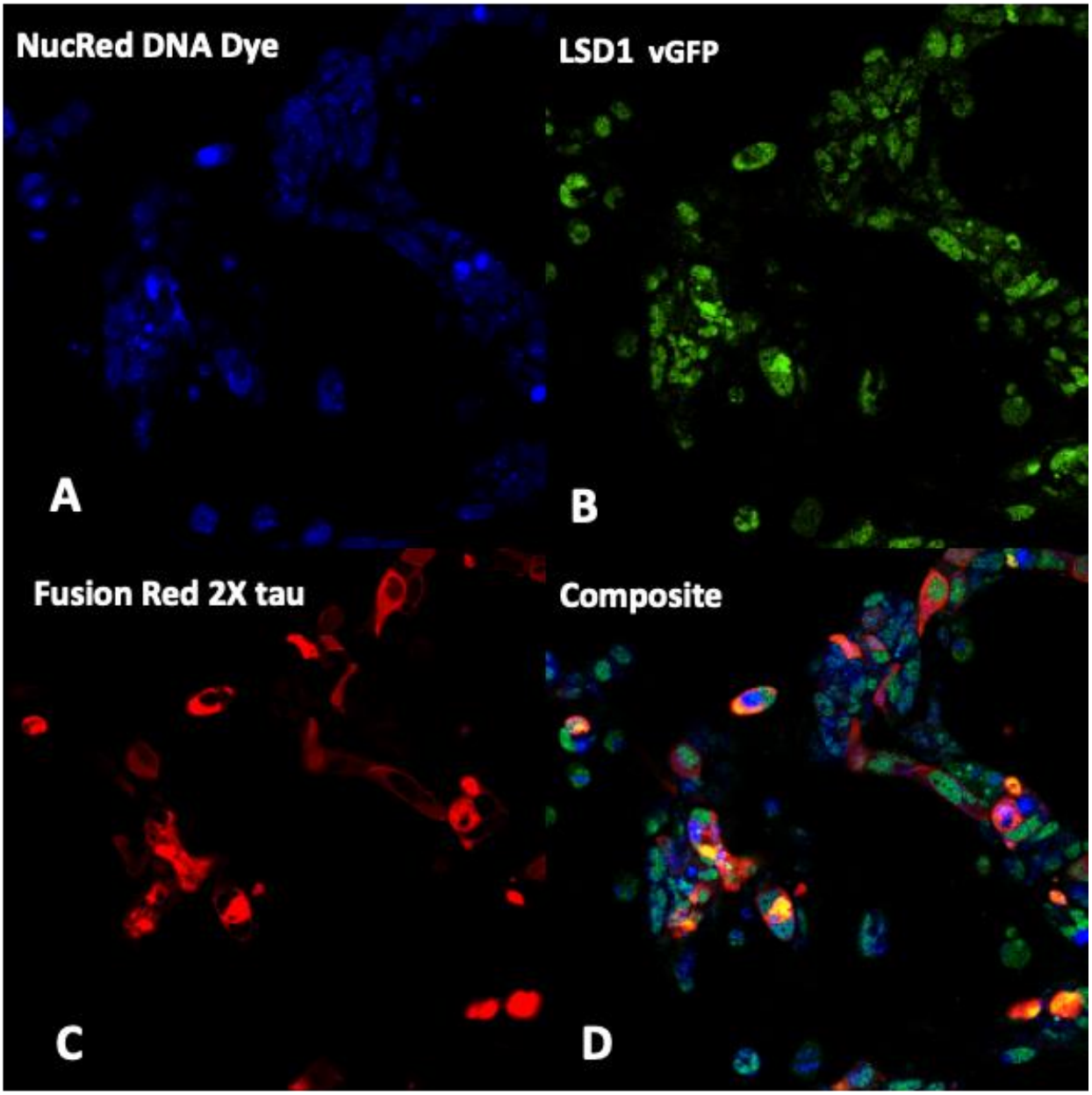


Figure 4. Tau transfection causes LSD1 to lose distinct nuclear enrichment. Cells treated with JetPrime transfection reagent and buffer 21 hours prior to imaging. . **A** NucRedDNA dye visualizes clear nuclei **B** LSD1 tagged with vGFP demonstrates LSD1 localizes to tau and loses nuclear enrichment. **C** Fusion red tagged 2X tau is not immediately expressed in all cells despite thymidine synchronization. **D** Merge image demonstrates LSD1 and DNA dye colocalization, non-nuclear LSD1 and Tau colocalization. **E1-4, F1-4** Zoom of A-D

However LSD1 clearly loses nuclear enrichment and localizes to tau (Figure 5E,F). However even cells that do not express tau at all or at high levels demonstrate altered morphology as demonstrated by cells with more diffuse LSD1 that colocalizes to the DNA dye(Figure 5D). Interestingly the most intensely enriched areas of LSD1 colocalize to tau

(Figure 5E,F). This demonstrates that LSD1 colocalizes to tau and prevents proper nuclear enrichment and supports the hypothesis that tau transfection causes defects in mitosis.

Figure 5. Tau transfection causes altered LSD1 morphology



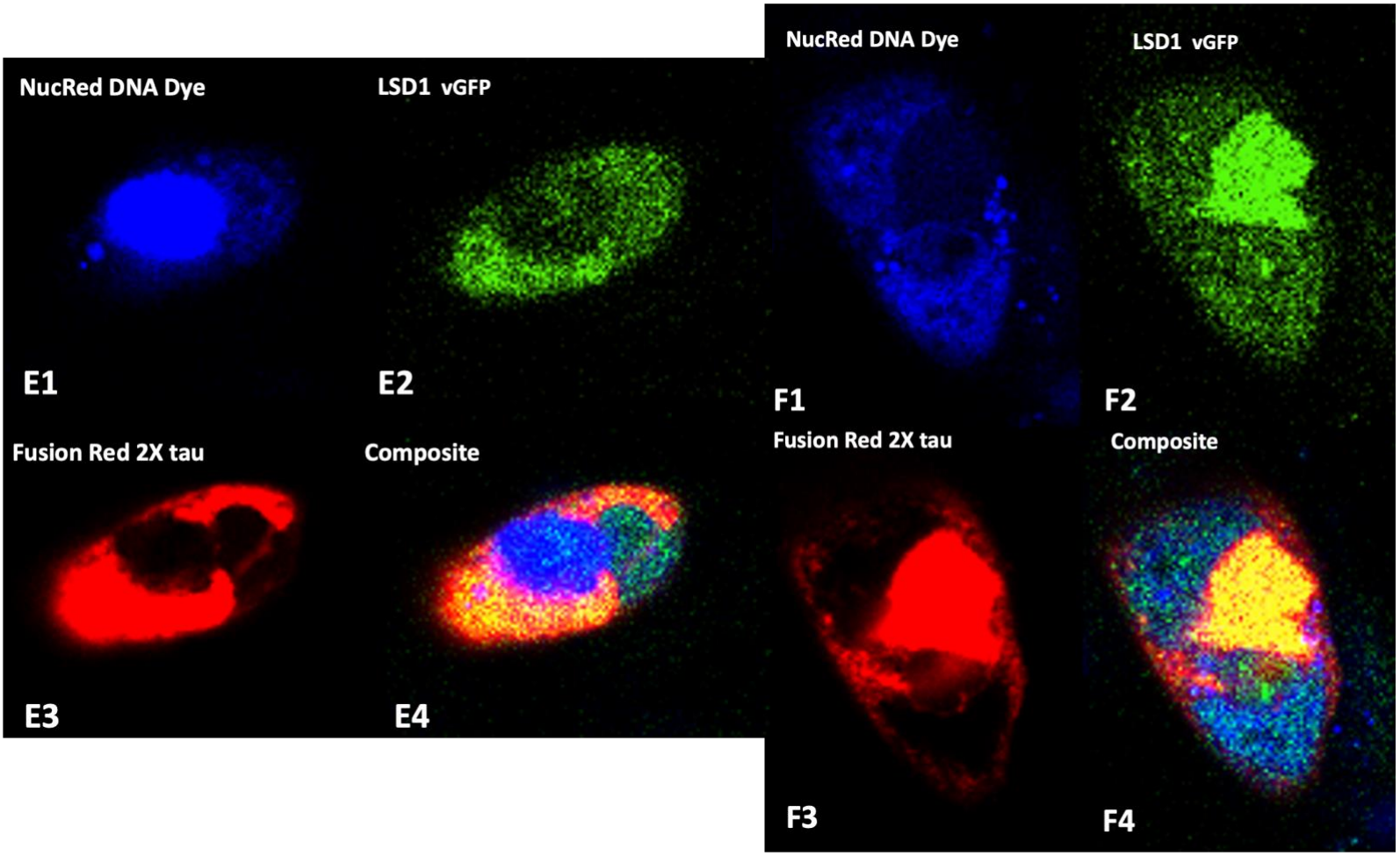


Figure 5 Tau transfection causes altered LSD1 morphology and localization Cells treated with JetPrime transfection reagent and buffer 21 hours prior to imaging.**A** NucRedDNA dye visualizes clear nuclei **B** LSD1 tagged with vGFP demonstrates LSD1 localizes to tau and loses nuclear enrichment. **C** Fusion red tagged 2X tau is not immediately expressed in all cells despite thymidine synchronization. **D** Merge image demonstrates LSD1 and DNA dye colocalization, non-nuclear LSD1 and Tau colocalization . **E1-4, F1-4** Zoom of A-D

After 36 hours after transfection there is much more global tau expression in almost all cells (Figure 6) and more cells demonstrate LSD1 colocalizing to tau and deficits in nuclear localization. In summary, these results demonstrate *in vitro* that LSD1 reliably colocalizes to tau and alters nuclear localization. These results will be foundational to a drug screen that attempts to block this interaction, and can be used in future experiments to elucidate the mechanisms behind the tau-LSD1 interaction

Figure 6. Increased tau expression causes LSD1 to lose distinct nuclear enrichment more globally

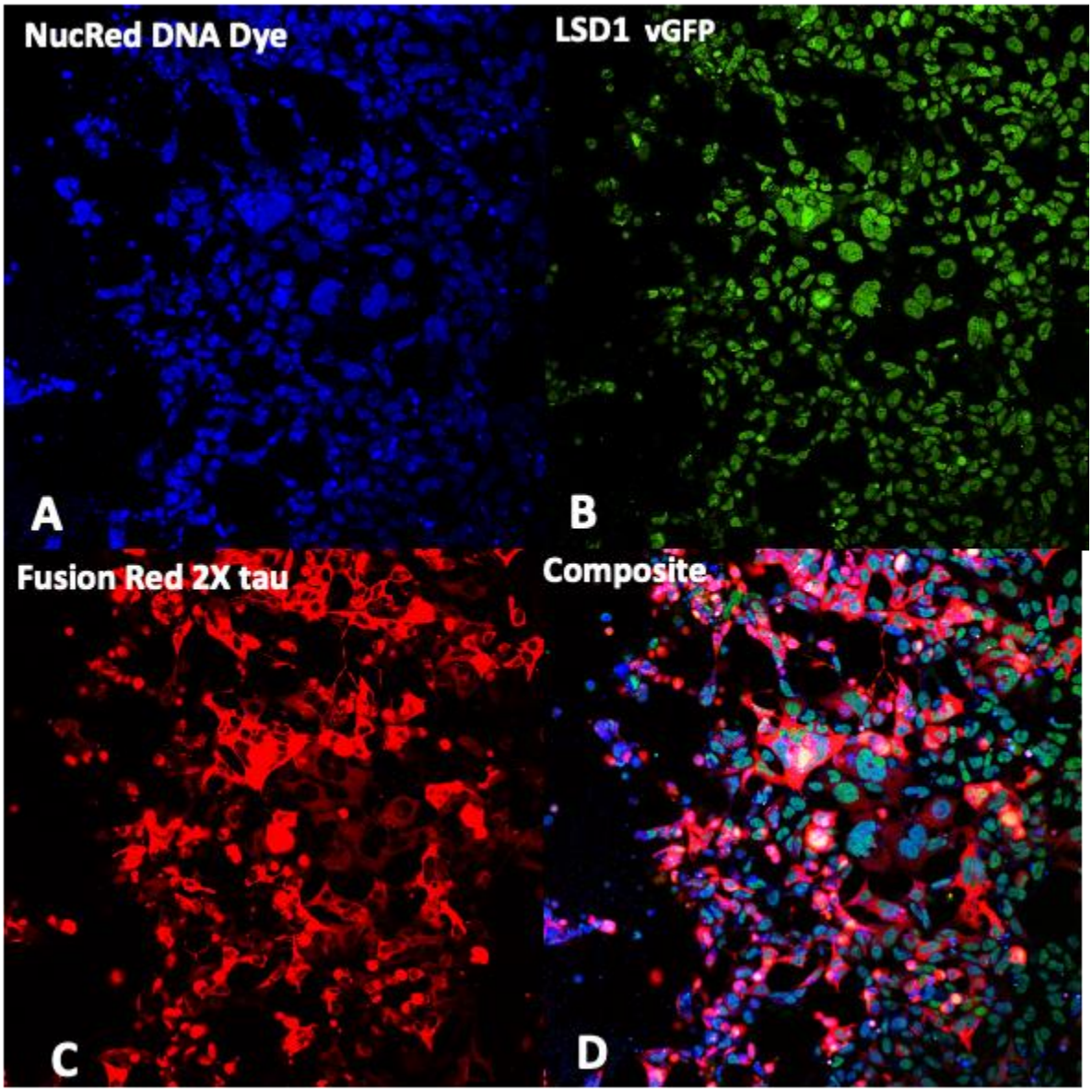


Figure 6. Increased tau expression causes LSD1 to lose distinct nuclear enrichment more globally. Cells treated with JetPrime transfection reagent and buffer 36 hours prior to imaging. **A** DNA dye **B** vGFP LSD1 **C** Fusion red tagged 2X tau is not immediately expressed in all cells despite thymidine synchronization. **D** Merge image demonstrates LSD1 and DNA dye colocalization, non-nuclear LSD1 and Tau colocalization .

However, despite this clear relationship, LSD1 does not directly bind to tau (Figure 7). Both antibodies are successful in precipitating their antigen but not the other protein. This suggests that LSD1 and Tau are not physically binding to each other but may be interacting by some other means such as electrostatic interactions. This result in combination with the clear colocalization and prevention of LSD1 nuclear localization supports the hypothesis that the disordered domains of the proteins are interacting and preventing proper LSD1 function.

Figure 7 Tau- LSD1 interaction is not strong enough to appear via immunoprecipitation

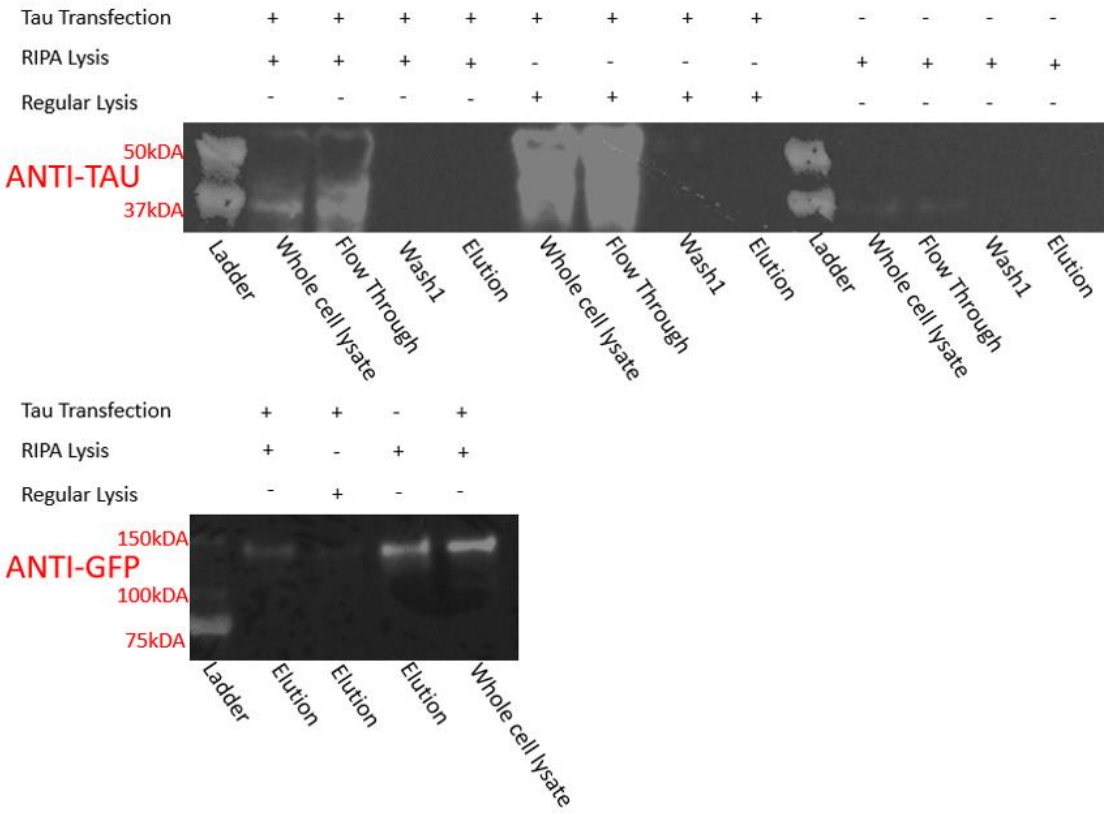


Figure 7 LSD1 does not physically interact with tau Western blot showing results of immunoprecipitation experiment in vGFP HEK293 cells mock transfected and transfected with tau. Two lysis buffers were also tested. Note: LSD1 is 110kDA, Tau is around 50 kDA

Image courtesy of Bai Yu.

Chapter IV

Discussion and Future Directions

Discussion and Future Directions

Our lab has previously shown neuronal nonnuclear LSD1-tau colocalization in human AD cases, replicated these findings in PS19 tau mouse models and demonstrated that LSD1 is continuously required for neuronal survival and LSD1 overexpression results in temporary rescue of neurodegeneration (Christopher et al., 2017, Engstrom et al., 2020). Here, I have recapitulated previous results by demonstrating tau sequesters LSD1 and prevents proper nuclear localization *in vitro* using HEK cells. Interestingly, LSD1 does not immediately lose nuclear enrichment. Instead LSD1 loses nuclear enrichment after mitosis when the nuclear envelope dissolves and LSD1 is exposed to cytoplasmic tau, and never regains nuclear enrichment before cell death.

Past research has shown that LSD1 is required for nuclear envelope reformation at the end of mitosis (Schooley et al, 2015) and future experiments can verify this nuclear envelope and mitotic deficit . While this specific finding is probably not incredibly related to neurodegeneration, it does demonstrate that when exposed to tau LSD1 normal localization is perturbed in a manner that may apply to cytoplasmic LSD1 in neurons before it reaches the nucleus. Furthermore, tau transfection alters the morphology of the nucleus as shown by the DNA dye stain in Figure 6 A,D.

There is some lack of uniformity in the expression of tau and mitosis despite synchronizing the cell cycle via thymidine block. This may be due to the increased stress of the tau transfection in combination with the thymidine block failing to maintain global cell cycle synchronization after release. Future experiments may try alternative methods to halt the cell

cycle closer to mitosis or attempting to wash cells with media instead of PBS to promote optimum recovery or attempt viral tau transfection.

While this model does not perfectly replicate the physiological environment of terminally differentiated neurons, this model can continue to investigate the interaction between LSD1 and tau in this scenario as LSD1 is produced in the cytoplasm where it can be exposed to pathological tau.. This project will provide the foundation for a drug screen to identify compounds that block the LSD1-tau colocalization demonstrated which may prevent tau-mediated neurodegeneration.

This methodology provides the means to answer mechanistic questions about the interaction between LSD1 and tau, such as the importance of the LSD1 NLS region and the IDD. The results from immunoprecipitation experiment support the hypothesis that LSD1 and tau's charged disordered domains are causing conformational changes that block the LSD1 NLS region. Future experiments to test this hypothesis, such as adding a crosslinker to the immunoprecipitation to test to see if LSD1 and tau are in proximity. These experiments are ongoing as a result of the preliminary data presented here. Additionally future experiments can also test whether tau is acting in a prion like manner towards LSD1. For example, LSD1-tau phase separation with and without a crosslinker would provide insight into whether tau is acting in a prion like manner towards LSD1 and causing a conformational change.

It would be of interest to test if proteins associated with other neurodegenerative diseases such TDP 43, alpha synuclein, and various isoforms of tau produce the same effect. Transfection with different plasmids that encode these proteins, we can continue to explore LSD1's role in neurodegeneration and how it interacts with these pathological proteins. Additionally by

transfecting cells with tau and a LSD1 plasmid that has a functioning NLS outside of the disordered domain, we can test whether this is the mechanistic site of action. Due to time constraints, these experiments are outside the scope of this thesis, but will continue to advance our understanding of the LSD1-tau interaction and LSD1's role in neurodegeneration more broadly.

Chapter V

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