9502 Learning Log

Class 1

October 7, 2024

Pre session Task

**Dr. Han**

Dr. Han's lab, who is a part of department of biochemistry

which is focused on unraveling the complex mechanisms behind fetal and placental growth, with a particular emphasis on understanding conditions like fetal growth restriction and preeclampsia.

Dr. Han’s team aims to uncover the molecular and cellular processes involved in these disorders, with the goal of improving maternal and neonatal health outcomes. the lab employs several cutting-edge approaches:

1. **Transgenic and Gene Targeting Technologies**: explores the roles of Insulin-like Growth Factor system in mice, examining how it influences fetal and placental growth through paracrine and endocrine signaling pathways.
2. **Gene-Environmental Interaction Models**: By manipulating nutritional and substrate supplies in pregnant animals, the lab studies how the environment and genetics interact during fetal and placental development, shedding light on developmental programming.
3. **Genomic and Proteomic Approaches**: These are used to investigate molecular mechanisms of fetal growth restriction and preeclampsia by analyzing biological materials such as plasma, amniotic fluid, and placentae from human subjects.

Dr. Han’s research ultimately seeks to **develop new diagnostic tools and interventions that will improve outcomes for women suffering from preeclampsia and for newborns with low birth weight**.

**Dr. Eric Arts**

I am excited to introduce you to the dynamic research taking place in Dr. Eric Arts' laboratory with a particular focus on **HIV-1 pathogenesis**, drug resistance, and molecular biology.

Dr. Arts’ team employs a range of **state-of-the-art imaging techniques** to investigate the cellular and molecular mechanisms that drive disease progression. This work not only aids in understanding how infections develop but also helps identify **effective antimicrobial agents** and create **diagnostic tools** to detect hidden reservoirs of pathogens—essential for early and accurate detection of infections.

A standout feature of the lab is its **global impact**, having established a satellite laboratory in Kampala, Uganda, and later moving to **Western University** to continue pioneering research in microbiology and immunology. Over the past 20 years, the lab has been a collaborative hub.

In terms of its scientific mission, the Arts Lab is committed to advancing knowledge in areas such as **HIV-1 evolution**, drug resistance, and the search for a cure and vaccine for HIV-1. Recent findings from the lab have deepened our understanding of the virus's molecular biology, paving the way for new therapeutic strategies.

**Dr. Vanessa Dumeaux**

I’d like to introduce to the fascinating research conducted in **Dr. Vanessa Dumeaux’s lab**, which is dedicated to understanding the intricate relationship between the **immune response** and the **gut microbiome** and how this interplay influences human health and disease.

The lab's current research focuses on two primary areas:

1. **Development of Computational and Genomic Methods**: Dr. Dumeaux’s team is creating advanced techniques to analyze microbial systems, particularly in the context of varying health conditions and drug treatments. These methods aim to provide deeper insights into how the gut microbiome functions and adapts to changes in health or treatment.
2. **Identification of Predictive Markers**: The lab is also working to identify specific **tumoral**, **systemic**, and **gut microbial markers** that can help predict the outcomes of cancer therapies. This research has the potential to revolutionize how we approach cancer treatment, providing personalized insights that could improve both prevention and diagnosis.

Class 2

October 9, 2024

Presession tasks

FUS Paper

Overview

* investigates how the RNA-binding protein FUS (Fused in Sarcoma) undergoes phase separation and how this process is influenced by post-translational modifications
  + arginine methylation
  + molecular chaperones like Transportin 1 (TNPO1)
* The goal is to understand the underlying mechanisms that regulate FUS's phase separation behavior and how disruptions in this process contribute to neurodegenerative diseases
* The study explores the following:
  + **Phase Separation of FUS**: The paper describes how intermolecular interactions, particularly cation-π interactions between C-terminal arginine and N-terminal tyrosines, drive the demixing or phase separation of FUS. This process creates liquid-like droplets that can transition to gel-like states.
  + **Methylation's Role**: Arginine methylation is shown to regulate the strength of cation-π interactions. Hypomethylation of FUS, observed in neurodegenerative diseases, promotes the formation of stable, gel-like structures that impair the function of ribonucleoprotein (RNP) granules.
    - Arg Methyl. Weakens the cation strength
    - Hypomethyl = strong cation strength = creates gel like = impair function of RNP = phase separation
  + **Molecular Chaperones**: The molecular chaperone TNPO1 helps reduce the phase separation and gelation of both methylated and hypomethylated FUS, rescuing normal protein synthesis in neuronal cells.

Introduction

* Phase separation 🡪 makes FUS form membrane free organelles (RNP)
  + Thes MFO take up, sequester, transport and release key RNA and protein cargo that regulate local RNA and protein metabolism 🡪 if goes away 🡪 trigger disease
* Figure 1
  + Salt + argine methyl. Influences FUS phase separation
    - High salt concertation
      * Hypo meth FUS form non spherical droplet 🡪 this indicates that the proteins condense into **non-spherical, irregular-shaped droplets**. This indicates a more solid-like or gel-like phase, suggesting a shift from a dynamic, reversible liquid droplet phase to a more static, pathological gel state
        + Protein is no longer smooth, aggregating into more rigid, irregular shapes.

This behavior is associated with high protein concentrations and is indicative of abnormal phase behavior, which can impair normal cellular function and lead to disease states.

* + - * Methyl FUS form spherical droplets

Results: FUS phase behavior in vitro is modulated by slat and FUS concentration

* The researchers explored how salt concentration and FUS protein levels affect the phase separation behavior of FUS protein in lab experiments (in vitro).
* FUS can shift between different physical states (dispersed, liquid droplets, or gel-like) depending on environmental factors.
* Temperature had little impact on FUS phase separation at normal protein concentrations
* Lower salt levels promote phase separation.
  + When salt concentration is reduced, FUS proteins quickly form small, round droplets.
* At higher concentrations of FUS protein, the formation of droplets happens faster and in larger amounts.
* The formed droplets can fuse together, indicating a liquid-like state
* Whether or not FUS proteins were tagged with fluorescent markers (EmGFP) did not change their phase separation behavior.
* FUS protein tends to form **liquid droplet**s more easily when **salt concentrations are low**, and this effect happens faster when there is **more FUS protein present**.

Hypomethylation of selected FUS arginine promotes phase separation

* The researchers investigated how reducing arginine methylation in FUS protein impacts its ability to phase separate.
* FUS proteins can undergo a chemical change called **hypomethylation** 🡪 increases phase separation and solidification
  + FUS proteins were treated with **AdOx**, a chemical that blocks the enzyme responsible for methylating arginine🡪 **hypomethylated FUS** 🡪 form more, smaller, and irregular shaped droplets 🡪 don’t fuse well
    - In contrast to methylated droplets 🡪 easily merges and maintained liquid like state
* Reducing methylation of FUS proteins leads to the formation of irregular, gel-like droplets that do not easily merge, contributing to abnormal protein behavior associated with diseases.

Cation interactions participate in FUS phase separation

* **Cation-π interactions** 🡪interactions between positively charged arginine and aromatic rings in tyrosine 🡪 bring protein close together 🡪play an important role in FUS protein phase separation.
* **Mutating Arginine’s and Tyrosines**:
  + When **arginine** is changed to **alanine** 🡪 phase separation is blocked 🡪meaning the FUS protein can’t form droplets.
  + Changing **tyrosines** to alanine also stops phase separation.
  + Replacing arginine with another positively charged amino acid (**lysine**) keeps phase separation intact.
  + Replacing tyrosine with **phenylalanine** also maintains the ability to form droplets.
* Adding extra arginine to the FUS protein increases phase separation, forming more gel-like structures.
* When they mixed the N-terminal and C-terminal fragments of FUS protein separately, phase separation happened briefly, but the droplets were unstable, showing the importance of the full protein for stable droplet formation.
* The strength of interactions between arginine and tyrosine (cation-π interactions) is essential for FUS proteins to form droplets, and when these interactions are disrupted, phase separation is blocked.

Figure 2

* The figure investigates how mutations in **arginine** and **tyrosines** influence the ability of FUS to undergo phase separation.
* It also explores how enhancing these interactions by adding more arginine impacts the shape and stability of the droplets formed.
* **Panel A**: Shows images of FUS protein with various mutations at different salt concentrations, highlighting how phase separation is either enhanced or reduced depending on the mutations.
  + **Row 1**: Shows normal **methylated FUS** undergoing phase separation and forming **spherical droplets** at lower salt concentrations. As the salt concentration increases, fewer droplets form.
  + **Row 2**: **Mutating arginine to alanine** disrupts cation-π interactions, **blocking phase separation** entirely. This means the protein doesn’t form droplets.
  + **Row 3**: **Converting arginine to citrulline**, which removes the positive charge, also prevents phase separation.
  + **Row 4**: **Mutating arginine to lysine**, which keeps the positive charge, allows phase separation to occur, showing that the positive charge is critical.
  + **Row 5**: **Mutating tyrosines to alanine** also reduces phase separation, indicating that the aromatic tyrosines are important for cation-π interactions.
  + **Row 6**: **Mutating tyrosine to phenylalanine**, which preserves the aromatic ring, maintains phase separation, showing that the aromatic nature of the side chain is crucial.
  + **Rows 7-10**: Increasing the number of **arginine** promotes stronger phase separation, even at higher salt concentrations, forming **gel-like, non-spherical droplets**. Adding a mutation that reduces the number of available tyrosines rescues normal spherical droplet formation.
* **Panel B**: A diagram shows how the turbidity (cloudiness) changes with different FUS mutations, indicating the extent of phase separation.
  + Turbidity measures how cloudy the solution becomes, indicating how much phase separation (droplet formation) has occurred
  + adding more arginine increases turbidity, meaning more phase separation, while **arginine-to-alanine** or **tyrosine-to-alanine** mutations reduce turbidity
* **Panel C & D**: Images and graphs provide information about the circularity (shape) of the droplets, indicating whether they are spherical or irregular.
  + C: This panel shows that **methylated FUS** forms **highly spherical droplets**, indicating a liquid-like state.
    - Mutations like **6R/K** still form spherical droplets, but constructs with more arginine form **non-spherical droplets**, which are gel-like and more solid.
    - Adding the **tyrosine-alanine mutation** reduces the irregular shapes and makes the droplets more spherical again, confirming that reducing cation-π interactions shifts the droplets back to a more liquid-like state.
  + D: The graph provides quantitative data on the shape (circularity) of the droplets. Higher circularity means the droplets are more spherical (liquid-like), while lower circularity indicates they are more irregular (gel-like).
    - Mutations like **6R/A** and **ncY/A** make the droplets more spherical, whereas constructs with extra arginine (**FUS +16R**) form irregular, non-spherical droplets.
* **Panel E**: Schematics illustrate how different modifications affect cation-π interactions.
  + This diagram explains how methylation of arginine weakens cation-π interactions by reducing the positive charge on arginine.
  + It also shows how converting arginine to citrulline, or alanine disrupts these interactions, while substituting lysine keeps phase separation intact because it preserves the positive charge.
* **Panel F**: Shows that cooperative interactions between the tyrosine-rich N-terminal domain and arginine-rich C-terminal domain are critical for phase separation.
  + This panel illustrates how the **N-terminal (tyrosine-rich)** and **C-terminal (arginine-rich)** domains of FUS work together to promote phase separation.
  + When these two parts are mixed together, they form droplets briefly, but the droplets are unstable without being part of the full-length protein.
* **Overall Key Findings from Figure 2:**
  + **Cation-π interactions** between arginine and tyrosine residues are crucial for phase separation.
  + **Disrupting these interactions** (e.g., by mutating arginine or tyrosine) stops phase separation, while **enhancing them** (e.g., by adding more arginine) promotes stronger and more stable phase separation.
  + **More arginine** led to the formation of **non-spherical, gel-like droplets**, which are more solid and could be linked to pathological conditions.
  + The balance between these interactions helps regulate whether FUS forms liquid-like droplets or more solid, pathological structures

Figure 3

* The aim of this figure is to show how FUS proteins behave in living cells when they are methylated or hypomethylated, and how altering the number of tyrosine or arginine residues affects the formation of FUS granules, which are related to the phase separation process.
* **Panel A**: Images showing how different forms of FUS behave in cells with respect to granule formation, particularly under conditions where arginine residues are hypomethylated (treated with AdOx) or in mutants with extra arginine or altered tyrosines.
  + When cells are treated with **AdOx**, causing **hypomethylation** of FUS, the number of cells with visible FUS granules increases significantly. This hypomethylation makes FUS more likely to form **granules**.
  + Cells expressing **mutant FUS with extra arginine** also show more granules, indicating that adding extra arginine increases phase separation and granule formation.
  + **Mutating tyrosines** to alanine (ncY/A) reduces the formation of granules, while mutating tyrosines to phenylalanine (ncY/F), which preserves the aromatic ring, restores normal granule formation.
* **Panel B**: Graph quantifying the dose-dependent increase in cells with FUS granules after AdOx treatment.
  + As the **AdOx dose increases**, the number of cells with **FUS granules** increases, indicating that **hypomethylation** promotes granule formation in a dose-dependent manner. This shows that when FUS proteins lose methylation, they are more likely to phase separate and form solid-like structures.
* **Panel C**: Graph showing how tyrosine mutations impact the number of cells with FUS granules.
  + **Normal FUS** shows moderate granule formation.
  + **ncY/A mutation** (which disrupts the cation-π interactions) significantly **reduces granule formation**, meaning that tyrosines are critical for phase separation and granule formation.
  + **ncY/F mutation** (which preserves the aromatic ring structure) shows similar granule formation to wild-type FUS, meaning the aromatic nature of tyrosine is important for maintaining normal phase separation.
* **Panel D**: Graph quantifying the effect of extra arginine on the formation of FUS granules in cells.
  + As the number of **extra arginine** increases, the number of cells with **FUS granules** also increases.
  + This indicates that more arginine promotes stronger cation-π interactions and thus enhance phase separation, leading to the formation of more **solid, gel-like granules** inside cells.
* **Panel E**: Graph showing the presence of RIPA-insoluble FUS (more aggregated, less soluble FUS) in different FUS mutations, indicating the extent of solid, gel-like structures.
  + Cells expressing **wild-type FUS** or **ncY/F mutant FUS** (which can undergo normal phase separation) have less RIPA-insoluble FUS, meaning they are forming more **liquid-like droplets**.
  + Cells expressing **hypomethylated FUS (HYPO FUS)** or **FUS with extra arginine** have **higher levels of insoluble FUS**, indicating that they form **solid-like or gel-like structures**, which are more pathological.
* **Overall Key Findings from Figure 3:**
  + **Methylation and Granule Formation**: Hypomethylation of FUS (through AdOx treatment) significantly increases the formation of FUS granules, which are related to phase separation. This means that the less methylated FUS is, the more likely it is to phase separate and form solid-like structures, which can be harmful in cells.
  + **Cation-π Interactions**: Cation-π interactions between **arginine** and **tyrosines** are essential for FUS phase separation. Disrupting these interactions (e.g., by mutating tyrosines or reducing arginine methylation) reduces the formation of FUS granules.
  + **Adding Extra arginine**: Increasing the number of **arginine** enhances phase separation, resulting in more granules and promoting the formation of solid-like structures in cells.
  + Figure 3 shows that **methylation** and the balance between **arginine and tyrosine interactions** control whether FUS proteins form **healthy droplets** or **harmful solid-like granules**. When there is less methylation or when extra arginine is added, FUS is more likely to form abnormal, gel-like structures inside cells, which can be linked to neurodegenerative diseases.

Arginine Cation-π Interactions Modulate FUS Phase Separation in Cells" section:

* **Cation-π interactions**: These are important molecular interactions between **arginine** (positively charged) and **tyrosine** (aromatic) in the FUS protein that help the protein separate into droplets within cells.
* When arginine is **methylated** (a chemical group is added to it), this weakens the cation-π interaction, reducing the ability of FUS to form solid granules.
* When the FUS protein is **hypomethylated** (less methylated, such as after treatment with AdOx), these cation-π interactions get stronger, leading to the formation of **more granules** in cells. These granules are more solid and don't easily dissolve.
* **Mutations**:
  + **Mutating tyrosine to alanine** (a different amino acid that can't form cation-π interactions) reduces FUS granule formation, meaning tyrosines are crucial for these interactions.
  + However, **replacing tyrosine with phenylalanine** (another amino acid that can still form cation-π interactions) restores granule formation, showing that the aromatic ring of tyrosine is important for the process.
* Adding **extra arginine** to the FUS protein increases granule formation in cells, suggesting that stronger cation-π interactions promote phase separation.
* Strong cation-π interactions between arginine and tyrosine lead to **more FUS granules** in cells, especially when the FUS protein is hypomethylated, which can result in abnormal solid structures that may contribute to diseases like ALS and FTLD.
* The interactions between **arginine** and **tyrosine** help FUS proteins form droplets in cells. If these interactions become too strong (like when there’s less methylation or more arginine), the FUS proteins form more **solid clumps**. This can lead to problems in the cell and is linked to neurodegenerative diseases.

TNPO1, but Not EWS or TAF15, Acts as a Molecular Chaperone for FUS

* **TNPO1** is a special helper protein called a **molecular chaperone**. It helps keep FUS proteins from clumping together too much or forming harmful solid structures (granules).
* When FUS is **hypomethylated** (lacks methyl groups), it tends to form **more solid granules** in cells. However, when TNPO1 is present, it **reduces these granules** and keeps the FUS proteins from clumping together as much.
* The study also looked at two other proteins, **EWS** and **TAF15**, which are similar to FUS. These proteins did **not** have the same effect as TNPO1. Even though they were found in the FUS granules, they didn't stop FUS from forming the harmful clumps.
* T**NPO1** is the only one that acts like a "helper" for FUS by preventing it from forming these **solid structures**. This helps the cells function better and might prevent disease.
* **TNPO1** helps FUS proteins **stay healthy** and stops them from forming harmful clumps, while other proteins like **EWS** and **TAF15** don’t help in the same way.

Biophysical Analysis of FUS Phase Separation Probed with Amyloidophylic Dyes" section:

* The researchers used **special dyes** (Thioflavin T and pFTAA) that stick to proteins when they form **solid, structured clumps** (called amyloids) to study FUS phase separation.
* **Thioflavin T (ThT)**: This dye didn’t work well with FUS. It didn’t stick to or light up the FUS droplets, even when they were forming solid-like structures, so it wasn’t useful for this experiment.
* **pFTAA dye**: This dye worked better. It **stuck** to the FUS proteins when they formed **solid, gel-like droplets**, especially when the FUS proteins were **hypomethylated** or had extra arginine. The more solid and clumpy the FUS became, the more the dye lit up.
* The dye also showed that **TNPO1** (the molecular chaperone) could stop these solid structures from forming, as it reduced the amount of dye that stuck to the FUS proteins.
* The researchers used **dyes** to see how solid and clumpy the FUS proteins got. One dye (pFTAA) showed that **hypomethylated FUS** formed more solid clumps, while **TNPO1** could prevent this from happening.

Structural Analysis of FUS Phase Separation by AFM-IR Nanospectroscopy" section:

* The researchers used a special technique called **AFM-IR nanospectroscopy**. This combines **Atomic Force Microscopy (AFM)** and **Infrared (IR) spectroscopy** to look at the **shape and structure** of FUS droplets at a very tiny, detailed level.
* **What did they see?**
  + **Methylated FUS** (normal FUS) formed **soft, round droplets** with a mix of different structures like coils and sheets. These droplets stayed liquid-like and flexible.
  + **Hypomethylated FUS** (with less methylation) formed more **solid, gel-like droplets** that had stiffer areas. Some parts of the droplet were still soft, but other parts became more solid, like a gel.
  + **FUS with extra arginine** (like FUS +16R) formed even **stiffer, more solid structures**, similar to the hypomethylated FUS, showing that more arginine led to stronger, more gel-like clumping.
* The analysis showed that **methylation** controls how FUS proteins change from **soft liquid droplets** to **solid, gel-like clumps**. Hypomethylated FUS or FUS with extra arginine becomes more solid and structured, which can lead to harmful protein clumping.
* they used a special tool to see how FUS proteins change from **soft, round droplets** to **stiff, solid clumps**. **Hypomethylation** or **extra arginine** made FUS more solid, while **normal methylation** kept it soft and flexible

**Figure 4: TNPO1 as a Molecular Chaperone for FUS**

* This figure shows how **TNPO1**, a special helper protein, can stop FUS from clumping into harmful granules.
  + **Top row (Panel A)**: Images show how different helper proteins interact with FUS in cells. FUS forms harmful **granules** when it is hypomethylated. TNPO1, however, stops these granules from forming, while other proteins like **EWS** and **TAF15** do not.
  + **Graphs (Panel B and D)**: They measured how much FUS clumped together in cells. When TNPO1 was present, **fewer granules** formed. However, when EWS or TAF15 were present, it didn’t change anything—FUS still formed harmful clumps.
  + Panel C
    - This part focuses on the **axon terminals** of neurons (the long extensions where signals are sent).
    - The images show that when **hypomethylated FUS** forms granules, they can accumulate in the **axon terminals**, which is harmful.
    - **TNPO1** prevents this granule buildup in axons, keeping the neurons healthy.
  + **TNPO1** acts as a protective helper by stopping FUS from forming too many granules, while other proteins don’t help.

**Figure 5: pFTAA Dye Shows Solid Clumps in FUS**

* This figure uses a special dye called **pFTAA** to see when FUS forms harmful **solid clumps** in cells.
* **What’s happening?**
  + **Top row (Panel A)**: Images show how much the pFTAA dye lights up when it sticks to solid clumps of FUS. The dye lights up more in **hypomethylated FUS** or when FUS has **extra arginine**, meaning these forms of FUS are more solid and harmful.
  + **Graphs (Panel A and B)**: The amount of pFTAA dye that lights up is measured. **Hypomethylated FUS** and FUS with **extra arginine** light up much more, showing they form **more solid clumps**. The dye also shows that **TNPO1** can reduce the solid clumps in hypomethylated FUS.
  + The dye shows that **hypomethylated FUS** or FUS with extra arginine forms harmful **solid clumps**. 🡪 more Beta sheets
  + However, **TNPO1** can prevent these solid clumps from forming.

**ADMA FUS: Homogeneous Spherical Liquid-like Structures with Low β Sheet Content**

* **ADMA FUS** is the normal, **methylated** form of FUS.
* When ADMA FUS separates into droplets, they are **round**, **soft**, and behave like **liquid**.
* These droplets have a **low number of β-sheets** (a type of structured protein folding), meaning they stay flexible and don’t solidify.
* In summary: **ADMA FUS** forms **round, liquid-like droplets** that are **soft** and **don’t clump** into solid structures.

**HYPO FUS: Heterogeneous Assemblies with Liquid- and Gel-like Condensates**

* **HYPO FUS** is the **hypomethylated** form of FUS, meaning it has less methylation.
* In this form, FUS forms **a mix of different structures**: some parts of the droplets are still **liquid-like**, while other parts become **stiffer and more gel-like**.
* These structures are **less uniform** and have more **β-sheets**, making them more solid than ADMA FUS.
* In summary: **HYPO FUS** forms **both liquid-like and gel-like clumps**. It’s less uniform and tends to get **stiffer** in some areas, making it more **solid**.

**FUS +16R: Stiff, Non-spherical Parallel β-Sheet-Rich Hydrogen-Bonded Assemblies**

* **FUS +16R** has extra **arginine residues** added, which increases the number of **cation-π interactions**.
* This form of FUS creates **stiff, irregular-shaped clumps** that are **non-spherical** (not round).
* These assemblies are rich in **parallel β-sheets**, meaning they are **highly structured** and **more solid**, forming a gel-like or stiff consistency.
* In summary: **FUS +16R** forms **stiff, non-round, solid clumps** that have lots of **structured β-sheets**, making them much more solid than normal FUS.

**Arginine Methylation Status Regulates Neuronal FUS RNP Granule Function**

* **Methylation** of **arginine** (a chemical change) helps control how FUS proteins form **RNP granules** (structures important for managing RNA) in neurons.
* **Normal methylation** allows FUS to function properly, but when FUS is **hypomethylated**, it forms too many granules that don’t work well.
* In summary: **Methylation** helps FUS regulate granule formation in neurons, and **low methylation** leads to dysfunction.

**Hypomethylated FUS Assemblies Impair Neuronal New Protein Synthesis**

* When FUS is **hypomethylated**, it forms **clumps** that **block protein synthesis** in neurons (the process by which cells make new proteins).
* This means **hypomethylated FUS** interferes with the ability of neurons to make new proteins, which is critical for their function.
* In summary: **Hypomethylated FUS** harms neurons by **blocking new protein production**.

**TNPO1 Rescues Impaired Protein Synthesis in Axon Terminals**

* **TNPO1** (a helper protein) can **rescue neurons** by stopping hypomethylated FUS from forming harmful clumps.
* When TNPO1 is present, it restores normal **protein synthesis** in neurons, even when FUS is hypomethylated.
* In summary: **TNPO1** helps **fix the problems** caused by **hypomethylated FUS**, allowing neurons to make proteins again.

**Cation-π Interactions and Cooperativity between N- and C-Terminal Domains**

* **Cation-π interactions** (a special type of molecular interaction) between the **N-terminal** and **C-terminal** parts of FUS help it form droplets through **phase separation**.
* These interactions bring the two parts of FUS together and make it easier for FUS to form **liquid droplets** that can switch between different forms.
* In summary: Special interactions between the two ends of FUS help it form the **right kind of droplets** for normal function.

**TNPO1 as a Chaperone in Non-nuclear Compartments**

* **TNPO1** works as a **chaperone** outside the nucleus (the control center of the cell) to help FUS stay in the **correct form** and prevent it from forming **clumps** in areas like axons (the long part of a neuron).
* TNPO1 helps **maintain the balance** of FUS in parts of the cell that handle RNA and protein production.
* In summary: **TNPO1** is a **helper** that keeps FUS **healthy** in parts of the cell outside the nucleus.

**Methylation as a Physiological and Pathological Regulator**

* **Methylation** acts as a **regulator** for FUS, controlling whether it forms normal, liquid-like droplets or abnormal, solid clumps.
* **Hypomethylation** (reduced methylation) of FUS can lead to **diseases** by causing FUS to form **sticky, harmful structures** in cells.
* In summary: **Methylation** helps keep FUS **functioning properly**, and **low methylation** can cause **disease**.

**FUS and FTLD**

* In diseases like **Frontotemporal Lobar Degeneration (FTLD)**, FUS proteins become **hypomethylated** and form harmful clumps in neurons.
* The clumping of **hypomethylated FUS** plays a role in the **progression of neurodegenerative diseases**.
* In summary: **Hypomethylated FUS** contributes to **neurodegenerative diseases** like FTLD by forming **harmful protein clumps** in the brain.

**Overall Takeaway:**

* **Methylation** is crucial for regulating FUS protein function. When FUS is **hypomethylated**, it can form harmful **clumps** that block important cell processes like **protein synthesis**, especially in neurons. **TNPO1** helps prevent these harmful effects by acting as a **chaperone** that keeps FUS in a healthy state. This process is linked to diseases like **FTLD**, where FUS clumping leads to **brain cell damage**.

**Figure 6: TNPO1 Prevents Hypomethylated FUS from Forming Harmful Granules**

* **Purpose**: This figure shows how **TNPO1** helps prevent **hypomethylated FUS** from forming too many harmful **granules** in neurons.
  + **Top row (Panel A)**: The images show neurons with **FUS granules**. When FUS is **hypomethylated**, it forms a lot of these granules, which can be harmful. But when **TNPO1** is added, the number of granules decreases, meaning TNPO1 prevents this harmful clumping.
  + **Graphs (Panels B & C)**: These graphs measure how much FUS forms granules in the **axon terminals** (the end of neurons). Without TNPO1, **hypomethylated FUS** forms a lot of granules, but with TNPO1, there are **fewer granules**.
* **Takeaway**: **TNPO1** helps prevent **hypomethylated FUS** from forming harmful granules in neurons, keeping the cells healthy.

**Figure 7: TNPO1 Restores Protein Synthesis in Neurons Affected by Hypomethylated FUS**

* **Purpose**: This figure shows how **TNPO1** helps neurons make **new proteins** (protein synthesis) when **hypomethylated FUS** is interfering with this process.
  + **Top row (Panel A)**: The images show neurons where protein synthesis is blocked by **hypomethylated FUS**. In these neurons, adding TNPO1 helps restore **normal protein production**.
  + **Graphs (Panel B)**: The graph shows the level of **new protein synthesis** in neurons. When FUS is hypomethylated, protein synthesis is **reduced**, but with **TNPO1**, protein production goes **back to normal**.
* **Takeaway**: **TNPO1** helps **rescue protein synthesis** in neurons that are affected by **hypomethylated FUS**, helping the neurons function normally again.

**Panel A: Protein Synthesis in Neurons with ADMA FUS**

* **What’s shown?**
  + This panel shows neurons that have **ADMA FUS** (the normal, methylated form) using a special method to track **new protein synthesis**.
  + **Green fluorescence** indicates areas where new proteins are being made.
* **Key Findings**:
  + **ADMA FUS neurons** are producing **a lot of new proteins** (bright green spots), showing that protein synthesis is functioning normally.

**Panel B: Protein Synthesis in Neurons with HYPO FUS**

* **What’s shown?**
  + This panel shows neurons with **hypomethylated FUS (HYPO FUS)**, which disrupts protein synthesis.
  + The green fluorescence is much **weaker** compared to Panel A.
* **Key Findings**:
  + **HYPO FUS neurons** produce **very few new proteins**, indicating that hypomethylated FUS is **blocking normal protein production**.

**Panel C: Protein Synthesis in Neurons with HYPO FUS + TNPO1**

* **What’s shown?**
  + This panel shows neurons with **hypomethylated FUS (HYPO FUS)** but with **TNPO1** added to the cells.
  + **Green fluorescence** increases compared to Panel B.
* **Key Findings**:
  + When **TNPO1** is added, **new protein synthesis is restored**. Neurons start making new proteins again, even in the presence of hypomethylated FUS.

**Panel D: Quantification of Protein Synthesis**

* **What’s shown?**
  + This graph provides **quantitative data** on the level of **new protein synthesis** in neurons from Panels A to C.
* **Key Findings**:
  + **ADMA FUS** has the **highest level** of protein synthesis (as expected in healthy conditions).
  + **HYPO FUS** shows a **significant drop** in protein production.
  + When **TNPO1** is added to HYPO FUS, protein synthesis is **restored**, close to the level seen with ADMA FUS.

**Panel E: FUS Granules in Axon Terminals of Neurons with HYPO FUS**

* **What’s shown?**
  + This panel focuses on the **axon terminals** (the ends of neurons that send signals). It shows neurons with **hypomethylated FUS** forming **granules** (clumps) in these regions.
* **Key Findings**:
  + **HYPO FUS** causes the **formation of clumps** in axon terminals, which disrupts neuron function and could block protein synthesis.

**Panel F: FUS Granules in Axon Terminals with HYPO FUS + TNPO1**

* **What’s shown?**
  + This panel shows neurons with **HYPO FUS** but with **TNPO1** added.
* **Key Findings**:
  + When **TNPO1** is added, the **granules are reduced**, meaning TNPO1 prevents the harmful clumping in the axon terminals, helping restore normal function.

**Panel G: Quantification of FUS Granules in Axon Terminals**

* **What’s shown?**
  + This graph quantifies the number of **FUS granules** in the axon terminals for different conditions (HYPO FUS and HYPO FUS + TNPO1).
* **Key Findings**:
  + **HYPO FUS** shows **many granules** in the axon terminals, but with **TNPO1**, the number of granules is **significantly reduced**.

**Panel H: New Protein Synthesis in Axon Terminals with HYPO FUS**

* **What’s shown?**
  + This panel looks specifically at **protein synthesis** in the **axon terminals** of neurons with **HYPO FUS**. The green fluorescence is **weak**, meaning there is very little new protein being made.
* **Key Findings**:
  + **HYPO FUS** reduces **new protein synthesis** in the axon terminals, which is harmful to neuron function.

**Panel I: New Protein Synthesis in Axon Terminals with HYPO FUS + TNPO1**

* **What’s shown?**
  + This panel shows the same axon terminals, but with **TNPO1** added to the neurons with **HYPO FUS**.
  + The green fluorescence returns, showing that **new protein synthesis is restored** in the axon terminals.
* **Key Findings**:
  + **TNPO1** rescues **new protein synthesis** in the axon terminals, even in the presence of hypomethylated FUS.

**Overall Takeaway from Figure 7 (Panels A to I):**

* **Hypomethylated FUS** disrupts **protein synthesis** and causes harmful **granules** to form in **axon terminals** of neurons, preventing the neurons from functioning properly.
* **TNPO1** acts as a **chaperone** that reduces FUS granules and restores **normal protein synthesis**, both in the cell body and the axon terminals. This means TNPO1 helps protect neurons from the harmful effects of hypomethylated FUS, allowing them to keep producing proteins and maintaining their function.

In simple terms, **Panels A to I** show that **TNPO1** helps fix the problems caused by **hypomethylated FUS**, stopping harmful clumps from forming and allowing neurons to **start making proteins again**

Side notes to remember:

* **Normal cellular function**: Phase separation is a normal process that helps proteins like FUS form **membrane-less organelles** that regulate key cellular functions such as RNA transport and protein synthesis.
  + In healthy conditions, phase separation results in **round, liquid-like droplets**. These droplets are dynamic and can fuse with each other, making them well-suited for cellular processes. This is the beneficial side of phase separation—it helps proteins perform their roles efficiently.
  + **In Disease conditions**, when phase separation becomes dysregulated (like when FUS is **hypomethylated** or mutated), it can result in the formation of **irregular, non-spherical droplets** that are more gel-like or solid. These **non-spherical droplets** can impair the normal function of the protein and lead to the formation of toxic protein aggregates.

**Methods.**

* FM
* AFM
* Spectroscopy

**Experimental Systems**

* In vitro: purified cells
* Cell lines: HEK 293, SHSY-5Y
* Neurons

Repeats/ Stats

* **Variant**
* **Biological replica**
* **Technical replica**

**RNA Paper**

Introduction:

* ALS (Amyotrophic Lateral Sclerosis) and FTD (Frontotemporal Dementia) are serious brain diseases where neurons progressively die.
* Both diseases are the abnormal clumping of a protein called TDP-43, which is normally involved in regulating RNA (a molecule essential for protein production).
* In these diseases, TDP-43 moves out of the nucleus and accumulates in the cytoplasm, forming harmful inclusions that contribute to neurodegeneration.
* While researchers have identified some genetic causes for these diseases, the exact mechanism behind TDP-43 clumping and how it leads to neuron death remains unclear.
* The goal of the research is to understand how TDP-43 forms these toxic clumps and find ways to stop them, potentially leading to new treatments for ALS and FTD.

Optogenetic Modulation of TDP-43 Inclusions:

* the researchers explain how they created a special tool that uses blue light to control when and where TDP-43 forms clumps in cells.
* They attached a light-sensitive component to TDP-43, allowing them to trigger the clumping process by shining blue light on the cells.
  + This helped them observe how TDP-43 moves and behaves in real-time. When exposed to blue light, the TDP-43 proteins left the nucleus and formed clumps in the cytoplasm, which resembled the harmful clumps seen in diseases like ALS.
  + This method gives scientists a way to study how TDP-43 clumps form and potentially find ways to stop them

Aberrant LCD Phase Transitions Drive the Formation of TDP-43 Inclusions:

* A part of the TDP-43 protein, called the LCD (low-complexity domain), can switch between liquid and solid states. Normally, this switching is reversible and helps proteins move and function inside cells.
* However, when something goes wrong, the LCDs stick together in an abnormal way, causing TDP-43 to form solid clumps, which are harmful.
* These clumps, or inclusions, are linked to diseases like ALS.
* The problem gets worse when mutations in TDP-43 cause the LCDs to stick together more easily, speeding up the formation of these toxic clumps. This clumping process contributes to the death of nerve cells.

RNA-binding Inhibits TDP-43 LCD Homo-oligomerization:

* TDP-43 normally binds to RNA, and this RNA-binding prevents TDP-43 from clumping together.
* The part of the protein that can form harmful clumps, called the LCD, is stopped from doing so when TDP-43 is bound to RNA.
* When the protein can't bind RNA properly (due to mutations or other reasons), the LCD part starts sticking together, causing the formation of toxic clumps.
* The study shows that RNA-binding is essential in keeping TDP-43 from forming these dangerous clumps, and without RNA, TDP-43 is more likely to become harmful

Impaired SG Recruitment Promotes Aberrant TDP-43 Phase Transitions:

* stress granules (SGs) are temporary structures that form in cells during stress to protect important proteins.
* Normally, TDP-43 is recruited to these stress granules, which helps prevent it from clumping.
* However, when TDP-43 loses its ability to bind to RNA, it can’t enter these protective stress granules.
* Instead, TDP-43 forms harmful clumps outside the granules, which are linked to diseases like ALS.
* So, the inability of TDP-43 to enter stress granules leads to more abnormal clumping, contributing to nerve cell damage.

Bait Oligonucleotides Rescue Aberrant TDP-43 Phase Transitions and Neurotoxicity:

* researchers describe how they designed special small molecules, called bait oligonucleotides, to stop TDP-43 from forming harmful clumps.
* These oligonucleotides work by binding to TDP-43 and preventing its abnormal phase transitions (where the protein switches between liquid and solid states).
* By stopping TDP-43 from clumping, these bait oligonucleotides also protect nerve cells from damage and death.
* This finding suggests that using these oligonucleotides could be a potential treatment to prevent neurodegeneration caused by TDP-43 in diseases like ALS

Discussion:

* TDP-43 clumping, known as proteinopathy, plays a key role in several neurodegenerative diseases like ALS and FTD.
* Researchers developed a new method using light to control TDP-43 clumping in cells, which helped them understand how these clumps form and lead to neuron damage.
* The study shows that RNA binding normally prevents harmful TDP-43 clumps from forming, but when TDP-43 can't bind to RNA, it clumps more easily, causing cell death.
* The findings suggest that targeting TDP-43 clumping with RNA or special molecules like bait oligonucleotides could prevent these toxic changes and protect nerve cells.
* The study also highlights that stress granules, which usually help protect cells, may not always prevent TDP-43 clumping.
* Overall, understanding how TDP-43 forms clumps and finding ways to stop this process could lead to new treatments for neurodegenerative diseases.

**Figure 1: Optogenetic Modulation of TDP-43 Inclusions**

This figure likely shows how blue light can control the formation of TDP-43 clumps in cells. It may have images of cells before and after light exposure, with more clumping (inclusions) after light stimulation. It would also likely show a comparison of cells with or without this light-sensitive system, proving that the clumping is a result of the light exposure.

**Panel A: Design of the Optogenetic System**

This panel may show how the optogenetic system is structured. Optogenetics involves attaching a light-sensitive protein (called **Cry2** in this study) to TDP-43, the protein involved in clumping in diseases like ALS. Cry2 responds to blue light by causing the TDP-43 proteins to clump together, allowing the researchers to control when and where the clumps form in cells.

* **Diagram of Cry2-TDP-43 Fusion**: A visual showing the fusion of Cry2 (the light-responsive part) with TDP-43.
* **Blue Light Stimulation**: It likely depicts how exposing cells to blue light triggers TDP-43 clumping.

**Panel B: Live Cell Imaging of TDP-43 Clumping**

This part of the figure could show actual images of cells before and after they are exposed to blue light.

* **Before Blue Light Exposure**: The cells probably show TDP-43 mostly in the **nucleus**, with no visible clumps.
* **After Blue Light Exposure**: You would likely see the TDP-43 moving out of the nucleus and forming **clumps (inclusions)** in the **cytoplasm**. The inclusion formation is the hallmark of TDP-43-related diseases.
* **Quantification**: There may also be a graph showing that cells exposed to blue light develop more inclusions over time compared to those that are kept in the dark, demonstrating that the light triggers the clumping process.

**Panel C: Light-Induced TDP-43 Clumping is Specific**

Here, the researchers likely want to show that the clumping is caused by the light and the fusion of TDP-43 to the Cry2 protein, not just by overexpression of TDP-43 alone.

* **Control Cells**: These could be cells with normal TDP-43 (without the Cry2 fusion) or cells expressing Cry2 alone. You would expect no clumping in these controls, even with blue light.
* **Clumping in Experimental Cells**: Only the cells with the Cry2-TDP-43 fusion should show clumping when exposed to blue light.

**Panel D: Biochemical Properties of TDP-43 Clumps**

This part likely focuses on analyzing the nature of the clumps formed by light-stimulated TDP-43.

* **FRAP (Fluorescence Recovery After Photobleaching)**: This technique might be used here to assess whether the clumps are solid or liquid-like. After shining a laser on a part of the TDP-43 clumps to bleach the fluorescence, the researchers check if the fluorescence returns (indicating liquid properties). If there’s no recovery, it suggests that the inclusions are solid and immobile.
  + **Control Panel**: Showing recovery of fluorescence in cells without clumps.
  + **TDP-43 Panel**: Showing no recovery in cells with light-induced clumps, indicating that these clumps are solid and difficult to dissolve, similar to what is seen in diseases like ALS.

**Panel E: Clumping Causes TDP-43 to Become Detergent-insoluble**

This panel may show that after TDP-43 forms clumps, it becomes **detergent-insoluble**, which is another hallmark of disease-related proteins.

* **Detergent Solubility Test**: Cells are lysed, and the soluble and insoluble parts are separated. The clumped TDP-43 should be found in the **insoluble fraction**. This is shown through **western blotting** or similar techniques, where TDP-43 in the insoluble fraction is detected after light exposure.

**Panel F: Clumped TDP-43 Shows Disease-Like Features**

Here, the researchers likely compare the characteristics of the TDP-43 clumps in their light-triggered system with what is seen in actual ALS/FTD patient tissue.

* **Markers of Disease**: The clumps could show typical signs like:
  + **Hyperphosphorylation**: A chemical modification found in disease-related TDP-43.
  + **p62 Co-localization**: p62 is a protein that often accumulates in cells with TDP-43 clumps, and its presence in the clumps suggests they mimic disease features.
  + **Ubiquitination**: This is another modification typically seen in disease-related TDP-43 clumps.

**Conclusion of Figure 1:**

Figure 1 demonstrates that the researchers successfully created a system where TDP-43 clumping can be controlled by light. This allows them to study how TDP-43 behaves, how these clumps form, and how they resemble the clumps seen in patients with neurodegenerative diseases. It also confirms that these clumps share biochemical properties with disease-related TDP-43 inclusions, including being solid, detergent-insoluble, and associated with certain markers.

By showing this, Figure 1 sets the stage for understanding the processes behind TDP-43 clumping and how this can be used to test potential therapies.

**Figure 2: Aberrant LCD Phase Transitions Drive TDP-43 Inclusions**

This figure probably demonstrates how the low-complexity domain (LCD) of TDP-43 drives the formation of clumps. It could include images or graphs showing how, over time, certain TDP-43 proteins go from liquid-like droplets to solid, harmful clumps. The figure may also compare normal TDP-43 to mutant forms that clump more easily.

**Panel A: Light-Induced Phase Separation of the TDP-43 LCD**

This panel likely shows that the LCD of TDP-43 can undergo **phase separation**—changing from a dispersed, liquid-like state into more condensed droplets in response to light.

* **Images of Cells Before and After Light Exposure**: The cells may have droplets forming after exposure to blue light, showing how the TDP-43 LCD phase separates into liquid-like droplets.
* **Control Cells Without Light Exposure**: These cells would likely not show phase separation, confirming that the light stimulation is causing the phase change.
* **Phase Separation Characteristics**: The droplets may appear as liquid-like, rounded structures that fuse together or split apart, confirming they are in a dynamic, liquid state.

**Panel B: Reversibility of Phase Separation**

This panel might demonstrate that the light-induced phase separation is reversible under normal conditions.

* **Light Exposure Followed by Dark**: It could show that once the blue light is turned off, the liquid droplets disappear or dissolve, indicating that the phase separation can reverse under normal circumstances.

**Panel C: Light-Induced Droplet Formation Depends on LCD Concentration**

Here, the figure likely shows that the amount of TDP-43 LCD in the cell determines how many droplets form.

* **Graph of LCD Concentration vs. Droplet Formation**: The graph would likely demonstrate that when the concentration of LCD is higher, more droplets form, suggesting that the concentration of TDP-43 is important for triggering phase separation.

**Panel D: Chronic Light Exposure Causes Solid-Like Inclusions**

This panel is likely focused on what happens when phase separation occurs repeatedly or for too long, which can lead to **solid, harmful clumps** rather than liquid droplets.

* **Images of Cells with Prolonged Light Exposure**: After long or repeated exposure to light, the previously liquid-like droplets turn into solid clumps. These solid clumps are similar to the ones seen in diseases like ALS, where TDP-43 inclusions are harmful to cells.
* **Graph of Droplet Maturation**: The graph might show that over time, more droplets become permanent, irreversible clumps, especially with repeated or prolonged exposure to light.

**Panel E: ALS-Linked Mutations Cause Faster Phase Transition to Clumps**

This panel likely shows that mutations in TDP-43 associated with ALS (such as M337V, Q331K, or A321V) cause the phase transition to happen faster and result in more harmful clumps.

* **Comparison of Wild-Type vs. Mutant TDP-43**: Images or graphs could show that mutant versions of TDP-43 undergo phase separation more quickly and more readily form solid, harmful inclusions than normal (wild-type) TDP-43.
* **Graph Showing More Persistent Granules with Mutant TDP-43**: It would likely show that cells with ALS-associated mutations develop more stable, permanent inclusions over time compared to normal cells.

**Panel F: Increased Size of Inclusions with Mutant TDP-43**

This panel might focus on the size of the inclusions, showing that the ALS-linked mutations not only cause faster clumping but also lead to **larger inclusions**.

* **Images of Larger Clumps in Mutant Cells**: Cells expressing mutant TDP-43 would have larger, more persistent clumps compared to those with normal TDP-43.
* **Graph Comparing Inclusion Size in Normal vs. Mutant TDP-43**: This would highlight how the size of the clumps increases significantly with mutations, suggesting that these mutations make TDP-43 more prone to forming large, toxic inclusions.

**Panel G: Chronic Light Exposure Leads to Disease-Related Features**

This panel probably shows that the solid clumps formed after prolonged light exposure share biochemical markers with the harmful TDP-43 clumps found in ALS and FTD patients.

* **Markers like Hyperphosphorylation and p62-Positive Staining**: The figure likely shows that these chronic clumps are positive for markers such as **hyperphosphorylation** (a chemical change often found in disease-related TDP-43) and **p62** (a protein that accumulates in TDP-43 clumps).
* **No Recovery After Photobleaching**: The clumps might show **no recovery in FRAP experiments**, indicating that they are solid and not dynamic or liquid-like, mimicking disease-like features.

**Key Message of Figure 2:**

Figure 2 shows that the **low-complexity domain (LCD)** of TDP-43 plays a crucial role in forming both liquid-like droplets and solid, harmful inclusions. Under normal conditions, LCD-driven phase separation is reversible, but with prolonged or repeated stress, it becomes irreversible and forms toxic clumps. Mutations in TDP-43, commonly linked to ALS, speed up this process and make the inclusions more persistent and larger. This supports the idea that abnormal phase transitions of TDP-43’s LCD are central to the development of neurodegenerative diseases like ALS and FTD.

**Figure 3: RNA-binding Inhibits TDP-43 LCD Homo-oligomerization**

This figure likely shows that when TDP-43 is bound to RNA, it does not clump. There may be comparisons of cells with normal TDP-43 (binding to RNA) versus those with a mutant version that cannot bind to RNA, illustrating that the mutant form clumps much more. There may also be visual data of RNA-deficient TDP-43 forming clumps faster.

**Panel A: RNA-Binding Prevents TDP-43 LCD Phase Separation**

This panel likely shows that the TDP-43 LCD (low-complexity domain) on its own can form droplets (phase separate) when exposed to blue light. However, when the RNA-binding regions of TDP-43 are included, this phase separation doesn't happen.

* **Images or Diagrams**: These would likely compare two conditions: TDP-43's LCD without the RNA-binding domains (which phase separates into droplets) and TDP-43 with RNA-binding domains, where droplets do not form.
* **Graph Comparing Droplet Formation**: The graph might show that the presence of RNA-binding regions drastically reduces or eliminates the formation of these droplets.

**Panel B: Mutations in RNA-Binding Domains Restore Phase Separation**

This panel probably shows that when mutations are introduced into the RNA-binding domains (to prevent RNA from binding), phase separation (droplet formation) happens again, even though the RNA-binding domains are present.

* **Mutant TDP-43 with Reduced RNA-Binding Ability**: Images may show that when TDP-43 is mutated (with specific mutations in the RNA-binding regions), phase separation happens, even though these regions normally prevent it.
* **Droplet Formation with RNA-Binding Mutations**: This likely includes comparisons between normal RNA-binding TDP-43 (no droplets) and the mutated form (which forms droplets). A graph might illustrate that mutations restore the ability of TDP-43 to form droplets, similar to the LCD alone.

**Panel C: RNA-Binding Inhibits TDP-43 Clumping via Liquid-Liquid Phase Separation (LLPS)**

This part likely demonstrates that the RNA-binding domains prevent the LCD from undergoing **liquid-liquid phase separation (LLPS)**, which leads to clump formation.

* **Normal vs. Mutant TDP-43 with and Without RNA-Binding**: This section probably shows images or graphs comparing the behavior of normal TDP-43 with intact RNA-binding and mutated TDP-43 that cannot bind RNA.
  + **Normal TDP-43**: Likely does not form clumps because the RNA-binding domains prevent LLPS.
  + **Mutated TDP-43**: Clumps or droplets form easily because the mutations stop RNA from binding, allowing the LCD to stick together.

**Panel D: Fusion of RNA-Binding Domains with LCD Blocks Clumping**

This panel may explore how the fusion of the RNA-binding regions to the LCD blocks clumping, and whether impairing RNA binding with specific mutations restores clumping.

* **Fusion Proteins**: It likely compares normal RNA-binding fusion proteins with those that are mutated to prevent RNA binding. In the RNA-binding-competent fusion protein, clumping doesn’t occur, while the mutated fusion protein allows clumping to happen.
* **Graph Showing Reduced Clumping with RNA-Binding Ability**: A graph could show that clumping is drastically reduced or absent when RNA-binding regions are active but increases when mutations block RNA binding.

**Panel E: RNA Prevents Pathological TDP-43 Inclusions**

This panel likely shows that adding **external RNA** to cells can reduce the formation of harmful TDP-43 clumps. It demonstrates how RNA acts as a protective factor, preventing the LCD of TDP-43 from sticking together abnormally.

* **Addition of RNA Reduces Clumps**: The images might compare cells with and without RNA added. In cells with added RNA, there are fewer or no TDP-43 inclusions, while in cells without extra RNA, more clumps form.
* **Graph Showing Reduced Clumping with RNA**: The graph could illustrate that as more RNA is added, the number of harmful clumps decreases significantly.

**Panel F: TDP-43 Clumps in Disease-Like Conditions Lack RNA**

This part of the figure likely shows that the harmful TDP-43 clumps found in diseases like ALS do not contain RNA, further supporting the idea that the absence of RNA leads to clumping.

* **Staining or Imaging of TDP-43 Inclusions in Patient Tissues**: The images might show tissue from ALS or FTD patients where TDP-43 clumps are present, and an RNA-detection method (like RNA fluorescent in situ hybridization or RNA FISH) shows that there’s little or no RNA in these clumps.

**Key Message of Figure 3:**

Figure 3 demonstrates that **RNA-binding prevents TDP-43 from forming harmful clumps**. When TDP-43 can bind RNA, the LCD is prevented from undergoing abnormal phase transitions and forming clumps. However, when mutations or other factors reduce TDP-43’s ability to bind RNA, the protein forms harmful inclusions. This suggests that RNA-binding is a key factor in keeping TDP-43 from clumping, and when RNA is missing or when TDP-43 loses its ability to bind RNA, it leads to the formation of toxic clumps seen in neurodegenerative diseases like ALS and FTD.

In summary, Figure 3 shows that **RNA acts as a safeguard** against TDP-43 clumping, and without it, TDP-43 is more likely to form the solid inclusions that are linked to brain cell death.

**Figure 4: Impact of RNA on TDP-43 Phase Separation and Aggregation**

This figure might illustrate how adding RNA can stop TDP-43 from forming clumps. It may have experiments where different amounts of RNA are added to cells, with graphs showing that more RNA leads to less TDP-43 clumping. There might also be images showing how TDP-43 behaves differently with or without RNA present.

**Panel A: RNA Prevents TDP-43 Liquid-Liquid Phase Separation (LLPS)**

This panel likely demonstrates that RNA can stop the TDP-43 protein from undergoing liquid-liquid phase separation (LLPS), which is a precursor to the harmful clumping (aggregation) of TDP-43.

* **Without RNA**: The panel may show that when TDP-43 is placed in a solution without RNA, the protein forms droplets (phase separates), representing the initial step of clump formation.
* **With RNA**: When RNA is added to the solution, the droplets don’t form, or much fewer droplets appear. This suggests that RNA binding prevents TDP-43 from transitioning into its clumped state.
* **Graph Comparing LLPS with and without RNA**: The graph would likely illustrate that adding increasing amounts of RNA reduces the number of TDP-43 droplets, confirming RNA’s protective effect.

**Panel B: RNA Concentration Inhibits TDP-43 Clumping in a Dose-Dependent Manner**

Here, the researchers probably explore how different amounts of RNA influence the ability of TDP-43 to clump.

* **Low vs. High RNA Concentration**: The images or graphs likely show that at low concentrations of RNA, TDP-43 forms more droplets (indicating that there isn’t enough RNA to fully stop clumping). At higher concentrations, the droplets are greatly reduced or absent, showing that more RNA leads to better inhibition of phase separation.
* **Dose-Response Curve**: A graph would likely plot RNA concentration against the amount of TDP-43 clumping, showing a clear decrease in clumping as more RNA is added.

**Panel C: Aggregation of TDP-43 without RNA**

This panel likely shows what happens when RNA is absent—TDP-43 transitions from liquid-like droplets to solid, harmful clumps (aggregates).

* **Without RNA**: The images probably depict TDP-43 forming solid aggregates, which are similar to the toxic clumps seen in neurodegenerative diseases. These clumps are no longer dynamic (fluid-like) but are stable and harmful to cells.
* **Graph of Aggregation Over Time**: A graph might track the time it takes for TDP-43 to transition from liquid droplets to solid clumps. The data would likely show that without RNA, TDP-43 quickly forms these solid aggregates.

**Panel D: RNA Delays or Prevents TDP-43 Aggregation**

This panel likely shows that adding RNA to TDP-43 not only prevents phase separation but also slows down or stops the protein from forming solid aggregates.

* **With RNA**: Images might show that even after a long time, TDP-43 doesn’t form solid clumps if RNA is present. The RNA prevents the harmful aggregation from occurring.
* **Aggregation Kinetics**: A graph might compare TDP-43 aggregation over time with and without RNA. Without RNA, TDP-43 quickly forms aggregates, but with RNA, aggregation is either delayed or completely inhibited.

**Panel E: RNA’s Protective Effect is Reversed by RNase Treatment**

This part of the figure likely demonstrates that removing RNA from the solution causes TDP-43 to start clumping again. The researchers probably add **RNase**, an enzyme that breaks down RNA, to see what happens to TDP-43.

* **With RNase (RNA Removed)**: Images or data might show that after adding RNase, TDP-43 starts forming solid clumps because the protective RNA is gone. This suggests that RNA is actively preventing the clumps from forming, and when RNA is removed, TDP-43 can clump again.
* **Graph Showing Reversal of RNA's Effect**: A graph could track how TDP-43, which was stable with RNA, begins clumping again once the RNA is degraded by RNase.

**Panel F: Expression of RNA-Binding Deficient TDP-43 Causes Clumps in Cells**

This panel likely demonstrates that when TDP-43 loses its ability to bind RNA, it starts forming clumps even inside living cells.

* **Normal TDP-43 vs. Mutant (RNA-Binding Deficient) TDP-43**: The images probably show that normal TDP-43, which can bind RNA, doesn’t form harmful clumps. However, mutant TDP-43 that can’t bind RNA forms large, solid clumps in the cell’s nucleus or cytoplasm.
* **Comparison of Aggregation**: There might be graphs comparing the clumping behavior of normal and mutant TDP-43 in cells. The mutant, RNA-binding deficient TDP-43 likely forms aggregates much more frequently.

**Panel G: RNA-Binding Deficient TDP-43 Forms Inclusions with Disease-Like Features**

This panel likely shows that the clumps formed by RNA-binding deficient TDP-43 share similar features to the toxic inclusions seen in diseases like ALS and FTD.

* **Markers of Toxic Inclusions**: The panel might include staining for markers such as hyperphosphorylation and p62, which are commonly found in disease-related TDP-43 inclusions. Cells with RNA-binding deficient TDP-43 likely show positive staining for these markers, confirming that the clumps formed in these experiments resemble those seen in ALS/FTD patients.
* **FRAP (Fluorescence Recovery After Photobleaching)**: FRAP might be used to demonstrate that these inclusions are solid and don’t recover after photobleaching, indicating that they are stable and harmful, similar to what is seen in diseased neurons.

**Key Message of Figure 4:**

Figure 4 demonstrates that **RNA prevents TDP-43 from clumping and forming toxic inclusions**. When RNA is present, TDP-43 is kept in a liquid-like, non-toxic state. Without RNA, or when RNA is degraded, TDP-43 quickly forms harmful solid clumps. Additionally, mutant TDP-43 that cannot bind RNA behaves abnormally, forming clumps that resemble those found in neurodegenerative diseases like ALS and FTD.

In summary, RNA plays a crucial role in preventing TDP-43 aggregation, and without it, TDP-43 becomes prone to forming the toxic inclusions associated with neuronal death in neurodegenerative diseases. This suggests that maintaining proper RNA binding could be a potential therapeutic strategy to prevent or slow down diseases involving TDP-43.

**Figure 5: Impaired SG Recruitment Promotes Aberrant TDP-43 Phase Transitions**

This figure likely shows how TDP-43 fails to enter stress granules (SGs) when it can’t bind to RNA. It may compare images of cells with normal TDP-43 (that goes into SGs) versus mutant TDP-43 (which stays outside of SGs and forms clumps). There might also be charts showing differences in the behavior of TDP-43 in these two scenarios.

**Panel A: Normal TDP-43 is Recruited to Stress Granules (SGs)**

This panel probably shows that, under normal conditions, TDP-43 gets recruited to stress granules when cells are under stress. This prevents TDP-43 from forming toxic clumps.

* **Images of Cells Under Stress**: The images likely show TDP-43 moving into stress granules (highlighted by specific SG markers such as G3BP1) after the cells are exposed to stress (like heat shock or chemical stress from sodium arsenate).
* **Co-localization of TDP-43 and SG Markers**: The TDP-43 proteins are probably shown as co-localizing with SG markers, which means that they are properly entering the protective SGs.

**Panel B: RNA-Binding Deficient TDP-43 is Excluded from SGs**

This panel likely shows what happens when TDP-43 loses its ability to bind RNA—it can no longer enter stress granules.

* **Images of Mutant TDP-43 Under Stress**: In this case, cells with mutant TDP-43 (which can't bind RNA) likely show that TDP-43 is **excluded from SGs**. Instead of co-localizing with SG markers, mutant TDP-43 forms clumps outside the stress granules.
* **Comparison of SG+ and SG- Granules**: The panel might show two types of granules—those that are SG-positive (TDP-43 inside stress granules, indicated by co-localization with SG markers) and SG-negative (TDP-43 outside SGs, forming harmful clumps).

**Panel C: TDP-43 Granules Without RNA are Larger and More Static**

This panel likely focuses on the fact that when TDP-43 cannot bind RNA and is excluded from SGs, it forms larger, more permanent, and harmful inclusions.

* **Images or Graphs Showing Larger Clumps**: This part may show that TDP-43 outside SGs forms **larger and more persistent clumps**. These TDP-43 granules likely lack mRNA (as SGs usually contain RNA), and they mature into more solid, irreversible inclusions.
* **Size Comparison of SG+ vs. SG- Granules**: The figure might include a graph showing that the granules containing RNA (SG+) are smaller and more dynamic, while those that lack RNA (SG-) are larger and more static.

**Panel D: FRAP Analysis Shows that SG- TDP-43 Granules are Immobile**

This part of the figure likely uses **FRAP (Fluorescence Recovery After Photobleaching)** to show that TDP-43 outside of SGs is solid and immobile, while TDP-43 inside SGs remains dynamic and liquid-like.

* **FRAP for SG+ Granules**: In SG+ granules (where TDP-43 is inside the stress granules), the fluorescence probably recovers after photobleaching, showing that these granules are still fluid and dynamic, meaning TDP-43 is not stuck in an aggregated state.
* **FRAP for SG- Granules**: For SG- granules (where TDP-43 is excluded from stress granules), there likely is no recovery after photobleaching, indicating that these granules are solid and permanent.

**Panel E: SG- Granules Lack RNA and Show Disease-Like Features**

This panel probably demonstrates that the harmful TDP-43 clumps that form outside of SGs are devoid of RNA and share features with disease-related inclusions seen in ALS and FTD.

* **Images Showing No RNA in SG- Granules**: RNA detection methods (like RNA FISH) might be used to show that the SG- granules formed by TDP-43 do not contain RNA. This absence of RNA allows TDP-43 to clump in a harmful way.
* **Staining for Disease Markers**: The TDP-43 clumps outside SGs might show positive staining for markers like **p62**and **hyperphosphorylation**, indicating that these granules are similar to the ones found in ALS and FTD patients' neurons.

**Panel F: RNA-Binding Deficient TDP-43 Forms Pathological Inclusions**

This part of the figure likely illustrates that the RNA-binding deficient TDP-43 forms **permanent inclusions** that resemble the pathological aggregates seen in neurodegenerative diseases.

* **Markers of Pathological TDP-43 Aggregates**: These inclusions likely stain for the same markers as those found in patient tissues, confirming that the TDP-43 granules forming outside of SGs are indeed harmful, disease-like inclusions.

**Panel G: Stress Granules Protect TDP-43 from Aggregation**

This final part likely sums up the role of stress granules by showing that **stress granules protect TDP-43 from clumping** and that losing this protection promotes harmful aggregation.

* **Diagram or Graph Summarizing the Results**: It could show how RNA binding is crucial for TDP-43’s recruitment to SGs and how, when TDP-43 is excluded from SGs (due to a lack of RNA binding), it forms large, static, and toxic inclusions that are similar to those seen in ALS and FTD.

**Figure 6: TDP-43 Inclusions Cause Neuronal Death**

This figure probably illustrates how TDP-43 clumps cause neuron death. It might show neurons before and after the formation of TDP-43 inclusions, with images showing neurons dying over time. There could be a graph tracking neuron survival, with more death in neurons that have TDP-43 clumps.

**Panel A: Expression of optoTDP43 in Neurons**

This panel likely shows the setup where neurons express **optoTDP43** (the light-sensitive TDP-43 fusion protein) and are subjected to light to induce the formation of TDP-43 inclusions.

* **Images of Neurons with optoTDP43**: The images may show neurons that have been genetically modified to express the light-sensitive TDP-43 protein. This setup allows the researchers to trigger clumping with blue light, making it easier to study the effects of these clumps on neurons.
* **Control vs. Light-Exposed Neurons**: Control neurons kept in the dark might look normal, while neurons exposed to blue light likely show TDP-43 inclusions in the cytoplasm.

**Panel B: TDP-43 Inclusions Cause Neuronal Death Over Time**

This panel likely demonstrates that when TDP-43 forms clumps in neurons (due to light exposure), it eventually leads to neuronal death.

* **Live Cell Imaging Over Time**: The images or videos likely track neurons over a period of time, showing how neurons with TDP-43 inclusions gradually lose their normal shape and die. Neurons exposed to blue light would show signs of damage, such as **blebbing** (cell membrane bulging) and shrinking, before eventually dying.
* **Graph of Neuron Survival Over Time**: A graph would likely show that neuron survival decreases significantly after prolonged light exposure, as more neurons die due to TDP-43 inclusions. In contrast, neurons that are kept in the dark (control) would have much higher survival rates.

**Panel C: Comparison of Neuronal Death Between optoTDP43 and Control**

This panel likely compares the effect of expressing optoTDP43 versus control (non-optoTDP43 or non-stimulated) in neurons.

* **Control Cells**: Neurons expressing just the Cry2 photoreceptor without TDP-43 likely show no signs of death, even with light exposure.
* **optoTDP43 Cells**: Neurons expressing optoTDP43 would have much higher levels of cell death after light exposure. A bar graph might compare the percentage of dead neurons in each group, with optoTDP43-expressing neurons showing a marked increase in death compared to controls.

**Panel D: Cytoplasmic Shift of TDP-43 Before Neuron Death**

This part probably illustrates that, before neurons die, the TDP-43 protein moves from the nucleus to the cytoplasm, where it forms harmful clumps.

* **Imaging Showing TDP-43 Movement**: Before the neurons die, TDP-43 may shift from its normal location in the nucleus to the cytoplasm, where it forms inclusions. This movement is a key step in the progression toward neurodegeneration.
* **Time-Lapse Images**: These images likely show the movement of TDP-43 over time, correlating with neuron damage and eventual death.

**Panel E: Neuronal Death is Associated with Large Inclusions and Small Particles**

This panel likely compares two types of TDP-43 assemblies: **large inclusions** and **smaller, more circular particles**, both of which are toxic to neurons.

* **Images of Neurons with Large Inclusions and Small Particles**: Neurons with either large TDP-43 clumps or smaller particles would both show signs of damage. These smaller particles might be an earlier or different form of TDP-43 aggregation.
* **Graph Showing Neuron Survival with Different Assembly Types**: A graph might show that neurons harboring either large inclusions or small particles have reduced survival compared to neurons with diffuse (unaggregated) TDP-43.

**Panel F: Time to Neuronal Death After Inclusion or Particle Formation**

This panel likely focuses on the timing of neuron death after the formation of TDP-43 inclusions or particles.

* **Graph Showing Time to Death**: The graph would likely plot how long it takes for neurons to die after forming TDP-43 inclusions or particles. It might show that once TDP-43 begins to aggregate (either as large inclusions or smaller particles), neurons die within a certain timeframe. There might be no significant difference in the time-to-death between neurons with large inclusions and those with smaller particles, suggesting both are equally toxic.

**Panel G: TDP-43 Inclusions Exhibit Pathological Markers**

This panel probably shows that the inclusions formed in these experiments share key biochemical features with those found in ALS/FTD patient tissues.

* **Hyperphosphorylation and p62 Markers**: Images or staining data might show that the large TDP-43 inclusions in neurons are positive for markers like **hyperphosphorylation** and **p62**, which are characteristic of TDP-43 pathology in neurodegenerative diseases.
* **Comparison of Inclusions and Particles**: While the large inclusions might show positive staining for these markers, the smaller particles may not, yet both are toxic to neurons.

**Key Message of Figure 6:**

Figure 6 demonstrates that **TDP-43 inclusions are highly toxic to neurons**, leading to cell death. Neurons exposed to light stimulation (through the optoTDP43 system) form large inclusions or smaller particles, both of which cause neurons to die over time. The movement of TDP-43 from the nucleus to the cytoplasm, followed by the formation of these toxic assemblies, is a key step in the progression to neurodegeneration. The figure also shows that these inclusions share similar features with the pathological aggregates found in diseases like ALS and FTD, confirming their relevance to real-world neurodegenerative conditions.

In summary, Figure 6 highlights the devastating effect of TDP-43 inclusions on neuron survival and provides insights into how these clumps drive neurodegeneration in diseases like ALS and FTD. It suggests that both large inclusions and small TDP-43 particles are equally harmful to neurons.

**Figure 7: Bait Oligonucleotides Reduce TDP-43 Clumping and Protect Neurons**

This figure likely demonstrates that adding bait oligonucleotides (small RNA-like molecules) reduces the clumping of TDP-43 and protects neurons from dying. There may be images showing fewer TDP-43 clumps in treated cells, along with graphs showing increased neuron survival when bait oligonucleotides are used.

**Panel A: Bait Oligonucleotides Reduce TDP-43 Clumping**

This panel likely shows how bait oligonucleotides effectively stop TDP-43 from clumping inside cells.

* **Images of Neurons with and without Bait Oligonucleotides**: The images might compare cells treated with bait oligonucleotides and those without. In untreated cells (without the oligonucleotides), you would likely see TDP-43 forming clumps or inclusions in the cytoplasm. In treated cells, fewer or no clumps would form, indicating that the oligonucleotides prevent aggregation.
* **Quantification of Clumps**: A bar graph or plot might show the number of TDP-43 inclusions in cells with and without oligonucleotide treatment, demonstrating a significant reduction in clumping in the treated cells.

**Panel B: Oligonucleotides Target Specific TDP-43 Sequences**

This panel likely explains that the bait oligonucleotides are designed to bind to specific regions of TDP-43 RNA, preventing it from aggregating.

* **Diagram of Oligonucleotide Binding**: This part may illustrate the structure of TDP-43 and show where the bait oligonucleotides are designed to bind. By attaching to key parts of the TDP-43 RNA or protein, the oligonucleotides prevent the abnormal clumping of the protein.
* **Graph of Binding Efficiency**: There may also be a graph showing the efficiency of different oligonucleotides in reducing clumping, with specific sequences working better than others.

**Panel C: Bait Oligonucleotides Reduce Neuron Death**

This part of the figure likely shows that neurons treated with bait oligonucleotides survive longer and are healthier compared to untreated neurons with TDP-43 inclusions.

* **Images of Neurons with and without Treatment**: Neurons without oligonucleotide treatment might show signs of degeneration (such as shrunken or dead cells) due to the formation of TDP-43 inclusions. In contrast, neurons treated with bait oligonucleotides are likely more intact, with fewer signs of stress or death.
* **Graph Showing Neuron Survival**: A graph might plot the survival of neurons over time, comparing untreated neurons (which would show increased cell death) with treated neurons (which survive longer due to reduced TDP-43 aggregation).

**Panel D: Time-Lapse of Neuron Health with Oligonucleotide Treatment**

This panel likely tracks neuron health over time, showing how oligonucleotide treatment affects the progression of neurodegeneration.

* **Time-Lapse Imaging**: Images taken over several days might show that neurons treated with bait oligonucleotides remain healthy for longer, while untreated neurons deteriorate rapidly due to TDP-43 aggregation.
* **Graph of Neuronal Degeneration Over Time**: The graph could show that, over time, neuron death is much slower in the presence of bait oligonucleotides compared to untreated controls, highlighting the protective effect of these oligonucleotides.

**Panel E: Bait Oligonucleotides Rescue Neurons from Aberrant Phase Transitions**

This panel likely focuses on how the oligonucleotides prevent the abnormal phase transitions of TDP-43, which are responsible for toxic clumping.

* **Images of Cells Before and After Oligonucleotide Treatment**: In cells without oligonucleotides, TDP-43 undergoes abnormal phase transitions, forming solid inclusions. After treatment with oligonucleotides, the images would likely show that TDP-43 either stays in a liquid-like, reversible phase or doesn’t form inclusions at all.
* **Graph Comparing Phase Transition Events**: A graph might compare the number of phase transitions (from liquid to solid) in treated versus untreated cells, showing fewer transitions in the presence of oligonucleotides.

**Panel F: Bait Oligonucleotides Block TDP-43 Neurotoxicity in a Dose-Dependent Manner**

This part likely demonstrates that the effect of bait oligonucleotides depends on the dose: higher doses lead to better protection of neurons from TDP-43 neurotoxicity.

* **Dose-Response Curve**: A graph might show that increasing concentrations of bait oligonucleotides lead to a stronger reduction in TDP-43 clumping and better neuron survival. The curve would illustrate that higher doses of the oligonucleotide provide greater neuroprotection.
* **Images of Neurons Treated with Different Oligonucleotide Doses**: The images could show neurons treated with low, medium, and high doses of bait oligonucleotides. Neurons treated with higher doses would likely show fewer signs of degeneration, while those with lower doses might still show some damage due to incomplete inhibition of TDP-43 clumping.

**Panel G: Bait Oligonucleotides Prevent TDP-43 Inclusion Formation**

This panel may show that neurons treated with bait oligonucleotides not only survive longer but also have significantly fewer TDP-43 inclusions, confirming the effectiveness of the treatment.

* **Images of Neurons with Fewer TDP-43 Inclusions**: Neurons treated with bait oligonucleotides likely show almost no TDP-43 inclusions, while untreated neurons show significant inclusion formation.
* **Comparison of Inclusion Formation**: A graph may compare the number of inclusions in treated versus untreated neurons, with oligonucleotide-treated neurons showing far fewer clumps, which correlates with improved cell health.

**Key Message of Figure 7:**

Figure 7 shows that **bait oligonucleotides can effectively prevent TDP-43 from forming toxic clumps** and protect neurons from the resulting neurodegeneration. These oligonucleotides work by binding to specific regions of the TDP-43 RNA or protein, blocking abnormal phase transitions and clump formation. Treated neurons exhibit higher survival rates, fewer inclusions, and a slower progression toward neurodegeneration. Importantly, the figure may also demonstrate that the protective effects of these oligonucleotides are dose-dependent, meaning that higher doses result in better prevention of TDP-43 neurotoxicity.

In summary, Figure 7 highlights the therapeutic potential of **bait oligonucleotides** as a treatment for neurodegenerative diseases like ALS and FTD, where TDP-43 inclusions play a central role in neuron death. By preventing these inclusions, the oligonucleotides could offer a way to slow down or even stop the progression of these diseases.

**Figure 8: Model of TDP-43 Phase Transitions and RNA-binding**

This figure is probably a diagram that summarizes the entire process described in the paper. It may show how TDP-43 forms clumps when RNA binding is reduced, leading to neuron death. It might also illustrate how RNA or bait oligonucleotides prevent TDP-43 from clumping, providing protection against neurodegeneration.

**Panel A: Normal TDP-43 Behavior with RNA-Binding**

This part likely illustrates how TDP-43 behaves under **normal conditions**, when it is properly bound to RNA.

* **Diagram of TDP-43 Binding RNA**: The diagram might show TDP-43 in its **normal state**—bound to RNA and located primarily in the **nucleus**. This prevents TDP-43 from forming harmful inclusions, keeping the protein in a functional, non-toxic form.
* **Phase Separation Prevention**: It likely emphasizes that RNA binding **prevents phase separation** and clumping of TDP-43, ensuring it stays in a healthy, dynamic state.

**Panel B: Abnormal Phase Transitions and TDP-43 Clumping Without RNA-Binding**

This section likely illustrates what happens when TDP-43 **loses its ability to bind RNA**, leading to **harmful phase transitions**.

* **TDP-43 Moving to Cytoplasm**: The diagram might show TDP-43 **leaving the nucleus** and moving to the **cytoplasm**, where it starts forming clumps due to the absence of RNA-binding.
* **Phase Separation into Liquid Droplets**: The model might show TDP-43 undergoing **liquid-liquid phase separation** (LLPS), forming liquid droplets as an early stage of aggregation.
* **Maturation into Solid Inclusions**: It could then depict how, over time, these liquid droplets mature into **solid, irreversible inclusions**, which are toxic to neurons. This process likely mimics what happens in diseases like ALS and FTD, where TDP-43 forms pathological clumps in the cytoplasm.

**Panel C: Role of Stress Granules (SGs) in TDP-43 Phase Transitions**

This part likely focuses on the protective role of **stress granules (SGs)** and how TDP-43 can normally be sequestered into these granules during cellular stress, preventing harmful aggregation.

* **TDP-43 Recruitment to SGs**: The diagram might show how, under stress conditions, **TDP-43 can be recruited into stress granules**, where RNA is abundant. This recruitment helps prevent TDP-43 from clumping into toxic inclusions.
* **Loss of RNA-Binding and SG Exclusion**: It may also highlight that when TDP-43 loses its ability to bind RNA, it **cannot enter stress granules** and remains in the cytoplasm, forming harmful aggregates instead.

**Panel D: Bait Oligonucleotides Rescuing TDP-43 from Clumping**

This section likely demonstrates how **bait oligonucleotides** can prevent the harmful phase transitions and clumping of TDP-43, offering a potential therapeutic strategy.

* **Bait Oligonucleotide Binding to TDP-43**: The diagram might show bait oligonucleotides binding to **specific regions of TDP-43**, preventing the protein from undergoing liquid-liquid phase separation and clumping. This binding mimics the natural protective effect of RNA.
* **Prevention of Inclusions**: The model could depict how, with the presence of bait oligonucleotides, TDP-43 stays in a **soluble, non-toxic state**, preventing the formation of solid inclusions.
* **Neuron Survival**: It might also illustrate that neurons treated with bait oligonucleotides are **protected from the neurotoxic effects** of TDP-43 aggregation, helping them survive longer and stay healthier.

**Panel E: Summary of TDP-43 Neurotoxicity and Protection Mechanisms**

This final panel likely provides a summary of the overall findings, visually tying together the processes that lead to TDP-43 neurotoxicity and how they can be counteracted.

* **Pathway Leading to Neuron Death**: The diagram may show the pathway that leads to neuron death, starting from the loss of RNA-binding in TDP-43, to phase separation, clump formation, and eventual **neuron death** due to toxic inclusions.
* **Therapeutic Intervention with Bait Oligonucleotides**: It likely contrasts this toxic pathway with a **therapeutic pathway**, where bait oligonucleotides intervene to stop TDP-43 clumping, thereby preventing neuron death and potentially offering a treatment for neurodegenerative diseases.

**Key Message of Figure 8:**

Figure 8 serves as a **summary model** that visually explains the key findings of the study:

* **Normal TDP-43 behavior**: TDP-43 remains healthy when bound to RNA, preventing harmful clumping.
* **Loss of RNA-binding leads to disease**: When TDP-43 can’t bind RNA, it undergoes phase transitions and forms toxic inclusions, contributing to diseases like ALS and FTD.
* **Stress granules protect TDP-43**: Under normal conditions, stress granules help sequester TDP-43 and protect it from aggregating, but this protection is lost when RNA-binding is impaired.
* **Bait oligonucleotides as a treatment**: Bait oligonucleotides can mimic RNA’s protective effect, preventing TDP-43 from clumping and offering a potential therapy to protect neurons from neurodegeneration.

**Summary of Figure 8:**

Figure 8 provides a **comprehensive overview** of how TDP-43 transitions from a healthy state to a toxic one, with and without RNA, and how **bait oligonucleotides** could intervene to prevent neurotoxicity. It explains the mechanisms behind TDP-43 aggregation and highlights a promising therapeutic approach to prevent or treat neurodegenerative diseases like ALS and FTD.

FUS in ALS

* FUS transfer from nucleus to cytoplasmic

RNA binding protein in ALS: TDP-43 and FUS

* Low complexity 🡪 mis folding
* RNA binding domain 🡪 RNA and protein

Aberrant RNA metabolism in ALS

Liquid phase separation

* 2 reagents (protein/ RNA)
  + Mix those it will self-organize 🡪 form droplets
* LLPS can still be functional but can enable the protein to go into disease state
  + If you can stop it here, you can prevent it
* Going from highly dynamic to a fixed point

LLPSP

* macromolecules
  + Between protein and protein RNA are important
* Driving forces
  + Poly ions
  + Cations
* Forms membrane organelles
  + Not harmful on its own
  + Not disease associate
  + Normal biological process

Class 3

October 16, 2024

Presession Tasks

* What does reproducibility mean?
  + Reproducibility refers to the **ability to obtain consistent results using the same methods and data as the original experiment or study.** It means that when someone else follows the **exact same procedures**, they should get the same or **very similar results.** Reproducibility is crucial in research because it ensures the reliability and validity of scientific findings.
* What does it mean if a result cannot be reproduced?
  + If a result cannot be reproduced, it indicates that others are unable to achieve the same findings when repeating the experiment using the same methods and data. This can raise questions about the **accuracy or validity of the original study,** and it could point **to errors in data collection, methodology, or even potential biases or misconduct**. In some cases, it might also highlight **complex factors** such as **variability in experimental conditions** that were **not adequately controlled.**
* What defines peer review?
  + Peer review is the process by which scientific work is evaluated by independent experts in the same field before it is published or accepted. The goal of peer review is to ensure the quality, accuracy, and validity of the research. Reviewers assess aspects such as the study design, methodology, analysis, and interpretation of results. Peer review helps to filter out flawed research and maintain scientific integrity by providing a critical assessment from experts who can identify weaknesses, errors, or areas needing improvement.
* What is the scale of the reproducibility problem?
  + The reproducibility problem is widespread and affects multiple fields of research, including medicine, psychology, biology, and economics. In some studies, particularly in the life sciences and social sciences, researchers have found that over half of published studies could not be reproduced. Surveys of scientists have shown that the majority have failed to reproduce the results of other researchers, and many have even been unable to replicate their own findings. This issue, often referred to as the reproducibility crisis, is seen as a major challenge in ensuring scientific rigor and progress.
* What are the societal and economic consequences of failing reproducibility?
  + Failing reproducibility has several societal and economic consequences:
  + Erosion of public trust: When scientific findings cannot be reproduced, it diminishes trust in science and research, which can lead to skepticism toward legitimate scientific advancements. This is particularly concerning in fields like medicine and public health, where inaccurate research can influence policy and healthcare decisions.
  + Wasted resources: Time, money, and effort are spent on research that cannot be replicated or verified. This represents a significant waste of public and private funding, especially when research is funded by governments, organizations, or donors.
  + Delays in scientific progress: Reproducibility issues can slow down innovation and discovery because resources are spent verifying and revisiting previously published studies, rather than building on solid, reliable research to make new advancements.
  + Health and safety risks: In fields like biomedical research, non-reproducible results can lead to flawed drug development, clinical trials, or medical treatments. This can have dire consequences for patient safety and healthcare outcomes if interventions based on flawed research are ineffective or harmful.

**Is there a reproducibility crisis in science?**

2011 Incident Example:

* Physicists observed neutrinos seemingly traveling faster than light.
* Error traced to a faulty fiber optic cable after thorough investigation.
* Emphasizes science as an ongoing, evolving process of discovery.
* Confidence in published results is crucial to avoid wasting resources and potential harm.

Reproducibility Challenge:

* Over a million scientific papers published yearly, but lack of resources to consistently verify findings.
* Studies show low reproducibility rates (e.g., less than 25% replication in pharmaceutical studies).
* Various sources of irreproducibility include:
  + Errors in study design, execution, or data analysis.
  + Uncontrolled variables (e.g., patient conditions).
  + Lack of transparency about original experimental procedures.

Systematic Issues in Science:

* "Publish or perish" culture drives pressure for significant, positive results.
* Researchers may have little incentive to challenge their own findings.
* Negative or inconclusive results are rarely published, skewing the literature.
* Peer review processes are sometimes insufficient, missing potential flaws in research.

Consequences of Irreproducibility:

* Wasted time, money, and resources.
* Erosion of public trust in science.
* Potential health and safety risks in fields like medicine.

Efforts to Improve Reproducibility:

* Push for more openness in sharing raw data, methods, and techniques.
* Strengthen peer review to better detect weaknesses.
* Encourage publication of studies that fail to confirm hypotheses.

Conclusion:

* Science naturally encounters false starts, but improving reproducibility will lead to more reliable discoveries and fewer wasted efforts.

**Is Most Published Research Wrong?**

2011 Study on Precognition:

* Published in the *Journal of Personality and Social Psychology*.
* Participants predicted the location of an image, with erotic images yielding a 53% hit rate.
* Result was statistically significant (p-value of 0.01), but there is skepticism about whether humans can predict the future.

Issue with p-values:

* p-values less than 0.05 are considered statistically significant.
* The 0.05 threshold is arbitrary, set by Ronald Fisher in 1925.
* Misuse of p-values leads to false positives in published research.

False Positives in Research:

* Published research often has a higher rate of false positives than expected.
* Even well-designed studies have false positives due to sampling, errors, and biases.
* Journals often avoid publishing studies with negative or non-significant results.

Reproducibility Crisis:

* Replication attempts show a low rate of reproducibility across various fields.
* In a Reproducibility Project, only 36% of psychology studies could be replicated.
* Cancer studies showed even lower reproducibility.

Sources of False Positives:

* Small sample sizes, multiple variables measured, and data manipulation (p-hacking) increase the likelihood of false positives.
* Researchers may make decisions that unintentionally lead to significant results (e.g., stopping data collection when reaching significance).

P-hacking and Biases:

* P-hacking: Manipulating data or analysis to achieve statistically significant results.
* The "publish or perish" mindset incentivizes researchers to find novel, significant results, sometimes at the cost of accuracy.

Replications and Journals:

* Replication studies are rarely published, and journals often reject replication attempts.
* Lack of replication contributes to the reproducibility crisis.

Improvement Efforts:

* Increased awareness of the reproducibility problem in recent years.
* Large-scale replication efforts, publicizing retracted papers (Retraction Watch), and repositories for negative results are helping.
* Pre-registered studies are gaining popularity, where methods are peer-reviewed before the experiment is conducted.

Conclusion:

* Despite its flaws, the scientific method is still the most reliable way of acquiring knowledge.
* Efforts are ongoing to address the reproducibility crisis and reduce false positives.

**Why Bad Science Spreads?**

Replication Crisis in Science:

* Multiple fields are struggling with replication, questioning foundational studies in psychology, biomedicine, and other areas.
* The crisis affects public policy, medical treatments, and the spread of misinformation (e.g., power posing).
* The problem is not just a few wrong findings, but the fact that errors went unnoticed for so long.

Explanations for the Crisis:

* Some deny the crisis; others blame weak standards like p-values.
* Factors include lack of replication incentives, biased institutions, and flawed statistical methods.

Natural Selection in Science:

* Science is subject to natural selection where traits (e.g., publication methods) are passed down through students, seminars, and papers.
* Selection in science favors productivity (number of papers, citations) over truth, leading to "gaming" the system (e.g., Goodhart’s Law).
* This results in sensational but unreliable findings (fool's gold), undermining long-term scientific progress.

Systemic Issues:

* Publication pressures (publish or perish) result in more research being published, often with exaggerated claims.
* Replication studies are undervalued, making it hard to correct false findings once they enter the literature.
* 85% of biomedical research is considered unreliable by *The Lancet*.

Cultural Impact:

* Increased use of terms like "innovative" and "groundbreaking" suggests pressure on scientists to produce novel results.
* Slower, more rigorous work is undervalued (e.g., Peter Higgs, Nobel laureate, says he wouldn’t be hired today due to low productivity).

Possible Solutions:

* Acknowledge the systemic issues rather than blaming individual scientists.
* Encourage incentives for replication, register hypotheses, and improve research quality.
* Re-align incentives towards truth, not just productivity, to reduce false positives.

Conclusion:

* Science, despite its flaws, remains the best method for discovering truth.
* Solutions include fostering deep, rigorous work and aligning incentives to prioritize replication and accuracy over quantity.

**1,500 scientists lift the lid on reproducibility**

* Focuses on the challenges of reproducibility in scientific research.
  + where many research findings, particularly in fields like biology, psychology, and medicine, cannot be reliably replicated.
  + This calls into question the robustness of some scientific studies.
* Highlights that many published results across various fields are difficult or impossible to replicate.
  + The article identifies several potential reasons for this, including inadequate statistical power, poor experimental design, and selective reporting of positive results. Researchers may inadvertently (or deliberately) report findings that align with their hypotheses, creating biases in the published literature.
* Stresses the importance of transparent methodology, robust data sharing, and the need for improved training for researchers.
  + A central theme is the growing advocacy for "open science" practices, where data, methods, and analysis codes are shared publicly to allow for greater transparency. This could enable other researchers to replicate findings and ensure that research is robust and trustworthy.
* Suggests incentives for reproducibility in academic publishing, including more journals emphasizing replication studies.
  + The article emphasizes the need for structural changes in the way science is done. Journals and funding bodies often prioritize novel findings over replications, which contributes to the crisis. It suggests that offering rewards and recognition for replication studies, along with better training for scientists in statistics and research methodologies, could help address this issue.
* Encourages the scientific community to adopt open science practices to enhance reliability and trust in research findings.
  + The scientific publishing system is critiqued for focusing more on the novelty of results than their reliability. It advocates for a shift in editorial policies where journals value replication efforts, potentially offering more outlets for these kinds of studies.

**Why Most Published Research Findings Are False**

* Examines selective publication and outcome reporting in clinical trials, emphasizing "publication bias."
  + where researchers may only publish positive or favorable outcomes from clinical trials, while negative or inconclusive results remain unpublished. This practice creates a bias in the medical literature, as the evidence base becomes skewed towards overly optimistic views of treatment effects.
* Reveals that many clinical trial results are not fully published or are selectively reported to emphasize positive findings.
  + Related to selective publication, outcome reporting bias occurs when specific outcomes are selectively reported or omitted from trial reports, depending on the nature of the results. For example, if a drug trial measures multiple outcomes, the ones that show positive effects may be highlighted, while those showing negative or neutral results are downplayed or ignored.
* Identifies that incomplete reporting can lead to skewed perceptions of a drug's efficacy or safety.
  + Physicians and policymakers may be misled by incomplete or selectively reported evidence, which could lead to the inappropriate use of treatments, misallocation of healthcare resources, or even harm to patients.
* Advocates for mandatory reporting of all clinical trial outcomes to increase transparency and accuracy in medical research.
  + To address these issues, the article advocates for full transparency in the reporting of clinical trial outcomes. It calls for mandatory registration of clinical trials (where all planned trials are logged in a publicly accessible database), along with full reporting of all results, regardless of their nature.
* Proposes measures like trial registries and full disclosure to address selective publication and improve accountability.
  + The article proposes that scientific and medical communities should implement strict requirements for reporting all trial data, including negative or inconclusive findings, in order to give a balanced view of a drug’s efficacy and safety. Additionally, it suggests that clinical trial registries (like ClinicalTrials.gov) could serve as repositories where all trial outcomes must be reported.

**Why Science Is Not Necessarily Self-Correcting**

* **Self-Correction in Science:**
  + **Traditional View**: Science is generally thought to be self-correcting, meaning that errors in research will eventually be identified and corrected through replication and scrutiny.
  + **Reality of Fluctuation**: However, Ioannidis argues that the trajectory of scientific credibility fluctuates, and there are no guarantees that the scientific record will always improve over time. Sometimes, credibility may even decrease.
* **Factors Impacting Scientific Credibility:**
  + **Unstable Trajectory**: The credibility of scientific findings can improve or worsen over time. It varies across different fields and is influenced by factors such as data availability, research methods, and replication efforts.
  + **Credibility Crises**: History has shown instances of major credibility failures, where entire fields or time periods experienced significant drops in scientific reliability.
* **Replication in Science:**
  + **Low Replication Rates**: Replication is essential for scientific self-correction, but many fields, including psychological science, rarely publish replication studies.
  + **Types of Replications**: Replications can either confirm a discovery or refute incorrect findings. However, direct replication by independent researchers is uncommon, and conceptual replications may reinforce original biases rather than correct them.
* **Discovery and Replication Paradigms:**
  + Ioannidis outlines several potential outcomes when a scientific discovery is tested:
  + **Optimal**: The original discovery is correct, and replication confirms it.
  + **Self-Correcting**: The original discovery is wrong, but replication corrects it.
  + **False Non-Replication**: The original discovery is correct, but replication fails due to errors in the replication study.
  + **Perpetuated Fallacy**: Both the original discovery and replication are wrong, leading to the continued propagation of false claims.
  + **Majority of Findings Unreplicated**: A significant portion of scientific findings remain unconfirmed because replication is so rare, leaving room for unchallenged fallacies.
* **Impediments to Self-Correction:**
  + Ioannidis lists several barriers that hinder the self-correcting mechanism in science:
  + **Publication Bias**: Positive or novel results are more likely to be published, while null or negative results are often disregarded ("file drawer" problem).
  + **Selective Reporting**: Researchers may selectively report results or analyses that align with their hypotheses, contributing to bias in the literature.
  + **Flexibility in Data Analysis**: The use of flexible, non-predefined methods in data analysis can lead to inflated or misleading results.
  + **Underpowered Studies**: Many studies are underpowered, meaning they do not have enough statistical power to detect true effects, which increases the likelihood of false positives.
  + **Editorial and Reviewer Bias**: Replications are undervalued by journals and peer reviewers, discouraging efforts to challenge existing findings.
* **Proposed Solutions to Improve Credibility:**
  + Ioannidis discusses several solutions to enhance the credibility of scientific research:
  + **Encourage Replication**: Increase the frequency and value placed on replication studies, particularly by independent researchers, to correct errors in the scientific record.
  + **Transparency**: Encourage open sharing of data, protocols, and analysis codes to allow others to verify results.
  + **Reform Incentives**: Shift the focus of science from producing novel, significant results to seeking the truth, by adjusting the incentive structures for publication and funding.
  + **Use of Students for Replication**: One suggestion is to involve students in replication efforts, but Ioannidis warns this could further diminish the perception of replication as valuable scientific work.
* **Challenges with Implementation:**
  + Ioannidis acknowledges that some of the proposed solutions, such as lowering publication standards or crowdsourcing replication, may have unintended consequences, such as flooding the literature with low-quality studies or reinforcing the idea that replication is trivial.
  + Overall, Ioannidis stresses the importance of maintaining the pursuit of truth as the primary goal of scientific research, rather than chasing novel or significant results. Without a strong commitment to transparency, replication, and rigorous methodologies, science risks losing its credibility.

**A long journey to reproducible results**

* Challenges in Reproducibility
  + Main Idea: The article highlights the difficulty of replicating scientific findings, especially in complex fields like aging research.
  + Elaboration: Researchers initially succeeded in extending the lifespan of *C. elegans* with a drug-like molecule. However, other labs were unable to replicate these results, creating frustration and confusion. The issue was not just with this experiment but reflected a broader problem of reproducibility in science. Variability between labs, including small differences in protocols, handling of worms, and experimental setups, contributed to discrepancies.
* Efforts to Understand Variability
  + Main Idea: Researchers tried to pin down the exact causes of variability in their results.
  + Elaboration: To improve reproducibility, scientists began to coordinate their methods in minute detail. They debated aspects like how to transfer worms between plates and how to handle reagents. Subtle differences, such as how forcefully technicians handled the worms or discrepancies in determining their age (based on hatching versus egg-laying), led to significant variation in lifespan results, highlighting how small methodological details can affect outcomes.
* Broader Implications for Biological Research
  + Main Idea: Reproducibility issues are widespread across biological research, not just in aging studies.
  + Elaboration: The article gives examples from other fields, such as cancer research, where differences in lab protocols led to inconsistent results. The need for detailed guidelines and standardized methods is emphasized, as even small deviations—like how samples are handled or stirred—can significantly impact results. This points to a broader need for rigorous standardization in biological research to improve consistency and reliability.
* Systematic Testing to Improve Replication
  + Main Idea: A systematic approach was developed to minimize differences between labs.
  + Elaboration: In response to replication challenges, the researchers launched the *Caenorhabditis Intervention Testing Program*, funded by the National Institute on Aging (NIA). They took meticulous steps to standardize every aspect of their experiments, from buying reagents with the same lot numbers to using identical incubators. By creating this uniformity, they were able to reduce variability across their labs and get more consistent results, although complete elimination of variability remained elusive.
* Unexpected Biological Findings
  + Main Idea: Variability in lifespan was not only due to lab conditions but also revealed underlying biological mechanisms.
  + Elaboration: After eliminating many methodological discrepancies, the researchers still noticed differences in lifespan among worms from the same genetic strain. This led to the discovery that worms could fall into either a short-lived or long-lived category, even under identical conditions. The reason for this variability is still unknown but suggests that intrinsic biological factors, rather than external conditions, can influence longevity, revealing new areas for further study.
* Genetic Diversity in Lifespan Studies
  + Main Idea: Genetic diversity plays a critical role in the outcomes of aging interventions.
  + Elaboration: The researchers tested multiple strains of *Caenorhabditis* species to explore how genetic background influences responses to compounds meant to extend lifespan. They found that some compounds worked across different strains, while others only worked in specific ones, and could even have negative effects in certain strains. This underscores the complexity of developing universal aging treatments and highlights the importance of genetic factors in the study of longevity.
* Standardization Versus Flexibility in Experiments
  + Main Idea: There’s a debate about the role of standardization in biological research.
  + Elaboration: While the researchers focused on creating uniform protocols to minimize variability, they acknowledged that some scientists argue for a more flexible approach. Instead of rigid standardization, some researchers advocate for testing whether results hold up under a variety of conditions. The article suggests that understanding the variability within a controlled environment is also valuable for uncovering biological insights.
* New Directions for Research
  + Main Idea: The research led to insights about the importance of describing methods in detail, not just results.
  + Elaboration: A key takeaway is that how experiments are conducted is as important as the results themselves. The authors argue that future research needs to focus more on describing the methods used in experiments to ensure reproducibility and better understand biological variability. This could help avoid situations where promising findings fail to replicate in other settings.

**Guiding questions**

* **Major Differences Between Scientific Journals and Non-Scientific Outlets:**
  + **Depth of Analysis**:
    - **Scientific Journals**: Articles in scientific journals go into deep detail regarding methodologies, experimental procedures, and data analysis. They are often highly technical, targeting an audience with expertise in the field. Each step of the research process is documented so that others can replicate the study.
    - **Non-Scientific Outlets**: Newspaper or popular media articles usually focus on summarizing findings in lay terms, with less emphasis on methodology or technical details. The focus is more on the impact or significance of the findings rather than on how they were obtained.
  + **Audience**:
    - **Scientific Journals**: Intended for a specialized audience of researchers, professionals, and experts in a particular field.
    - **Non-Scientific Outlets**: Aim for the general public, presenting information in a way that is easy to understand without specialized knowledge.
  + **Review Process**:
    - **Scientific Journals**: Articles are peer-reviewed, meaning they undergo a rigorous evaluation by other experts in the field before being published. This process ensures that the research meets the necessary scientific standards.
    - **Non-Scientific Outlets**: Articles are not peer-reviewed. Editors may review them, but the review process is not as stringent, and articles are published faster, with less scrutiny of the research details.
  + **Citations and Data**:
    - **Scientific Journals**: Articles contain extensive citations, references to previous research, and usually provide access to data or supplementary material. This is essential for validating the research and ensuring reproducibility.
  + **Non-Scientific Outlets**: Citations are less formal and often refer to general ideas or key figures rather than detailed data. The emphasis is more on storytelling or drawing attention to the societal relevance of the findings.
* **Commonalities:**
  + **Interest in Novelty**: Both scientific journals and newspapers share an interest in novel, groundbreaking findings. While scientific journals are more rigorous in validating claims, newspapers also focus on new, interesting research as it generates public interest.
  + **Communication of Impact**: Both types of articles aim to communicate the significance of research findings, though in different ways. **Newspapers focus on real-world implications for society, whereas scientific journals also discuss the broader scientific implications for the field.**
* **Reasons for Failed Reproducibility:**
  + **Methodological Differences**: Even small deviations in experimental design, procedures, or conditions (e.g., temperature, materials, equipment) can lead to different results. For example, as described in the roundworm research article, even subtle factors like how worms were handled or minor temperature fluctuations caused differences in lifespan outcomes
  + **Publication Bias**: Researchers and journals tend to favor novel, positive findings over negative or inconclusive results. As a result, negative replications (i.e., failures to replicate previous studies) may go unpublished, skewing the literature toward positive outcomes​
  + **Lack of Standardization**: In some fields, there is insufficient standardization of protocols across labs, making it difficult to compare results from different studies. This was a significant issue in aging research and other biological studies​
  + **Low Statistical Power**: Studies with small sample sizes or insufficient statistical power are more likely to produce false positives or results that can't be replicated. This is particularly common in fields like psychology, where underpowered studies can lead to exaggerated or non-replicable findings​
  + **Selective Reporting**: Researchers may selectively report only the outcomes that support their hypothesis, leaving out negative results or null findings. This leads to a distorted view of the evidence​
* **Fields More Affected by Reproducibility Issues:**
  + **Psychological Science**: This field has been highlighted as particularly affected by reproducibility problems. Studies in psychology often deal **with complex human behaviors**, and slight changes in experimental conditions can lead **to widely varying results**. The field has also been criticized for a high prevalence of false positives and selective reporting​
  + **Biomedicine and Preclinical Research**: Fields like cancer research, pharmacology, and aging research also face challenges with reproducibility. Biological systems are highly sensitive to minor changes in experimental conditions, and different labs may use slightly different protocols or equipment, leading to conflicting results​
  + **Social Sciences**: Similar to psychology, social sciences face reproducibility issues due to the complexity of human behavior and **societal variables**. Differences in sampling, context, and methodology between studies can make it difficult to replicate findings.
* **What Causes Bias in These Fields?**
  + **Pressure to Publish**: In academia, there is immense pressure to publish frequently, particularly in high-impact journals. This can lead researchers to prioritize novel findings over rigorous, replicable work. The tendency to publish only positive or significant results further exacerbates the problem​
  + **Funding and Incentives**: Funding agencies and institutions often reward groundbreaking discoveries rather than replication studies, creating a bias toward novel findings, even if they are less reliable
  + **Complexity of the Subject Matter**: In fields like biomedicine or psychology, the complexity of the systems being studied means that even slight variations in methodology can have large effects. The variability inherent in biological organisms or human behavior makes reproducibility particularly challenging

Lecture notes

Clinical vs Basic research

* Basic principles
* Differences and communalities
* Objectives
* Limitations

2nd

* Follow a certain hypothesis
* Hypothesis and discovery driven research are both important
* Experiment is for basic research
* Ethical conundrum
* Doing research properly comes with some ethical concerns
  + Placebo
  + Animal rights

3rd.

* Basic research
  + Knowledge advancement
  + Insight into mechanisms
  + Describe/ understand nature
* Clinical research
  + Test/establish
    - Medication
    - Device
    - Diagnostic tools
    - Treatments 🡪 confide to human subjects
      * Animal models are always basic research
        + Unless you do veterinary research
* A diagram of a health risk

  Description automatically generatedA diagram of a research process

  Description automatically generatedrequire thorough analysis

Clinical trial

* Gold standard
* Phase 1
  + Couple weeks to perform and couple months to analysis
  + Safety of disease
  + Check for side effects
  + Tested in healthy individuals
  + Consider diversity
    - Due to metabolic differences
  + Maybe 20
* Phase 2
  + Months to years
  + Efficacy of the treatment
  + Recruit people that have the disease
  + Test for benefit in sick people
  + Balance sex and gender, ethnicity y
  + At least 500
  + Rare disease will have a mix of phase 1 and 2 mixed together
* Phase 3
  + Combining phase 1 and 2
  + 1000s
  + Benefits of the drug
  + Assessing safety
  + Broader population
* Phase 4
  + Long term effect of certain therapeutic interventions
  + Multinational
  + Produces solid results

Basic procedure

* Randomization
* Placebo
* Double blind studies

A diagram of a treatment group

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Ethics of Clinical research

A screenshot of a computer

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Lecture 5

October 22, 2024

Presession tasks

DNA videos

**Restriction Enzyme Cloning**

* **Introduction**: Discusses the use of restriction enzyme cloning, a fundamental method for creating recombinant DNA.
* **Enzyme Example**: EcoR1, which specifically cuts the DNA sequence GAA TTC.
* **Process**:
  + Restriction enzymes cut DNA at specific sequences, creating "sticky ends."
  + These sticky ends facilitate the insertion of DNA fragments into plasmids.
* **Vector Preparation**: Uses a plasmid vector with a multiple cloning site (MCS) that includes sites for EcoR1 and other enzymes.
* **Digestion and Ligation**:
  + DNA and vector are cut by restriction enzymes.
  + DNA ligase is used to seal the DNA into the plasmid, creating a recombinant DNA molecule.

**DNA Ligase Function**

* **Role**: Catalyzes the formation of phosphodiester bonds between adjacent DNA fragments, essential for DNA repair and replication.
* **Mechanism**:
  1. **Self-Adenylation**: DNA ligase reacts with ATP or NAD to become adenylated.
  2. **Transfer of Adenyl Group**: The adenyl group is transferred to the 5' phosphate end of DNA.
  3. **Formation of Phosphodiester Bond**: The adenylated DNA end reacts with the 3' hydroxyl group of another strand to form a bond and release AMP.

**Gateway Cloning Technology**

* **Overview**: Describes Gateway cloning, a method based on site-specific recombination used to transfer DNA sequences between different plasmid backbones.
* **System Components**: Utilizes tat sites for recombination and enzymes like integrase and excisionase to manage DNA integration and excision.
* **Recombination Steps**:
  1. **Formation of Protein-DNA Complex**: Integrase binds to tat sites, aligning the DNA for recombination.
  2. **DNA Cleavage and Strand Swapping**: Integrase cuts the DNA, and the strands are swapped to form new connections.
  3. **Ligation and Release**: The DNA backbone is resealed, and the enzyme complex disassembles.
* **Application**: Allows the insertion of genes into different vectors for protein expression in various systems.

**Practical Applications and Considerations**

* **Directionality and Relegation Issues**: In restriction enzyme cloning, the orientation of the inserted DNA can be random, and unwanted re-ligation of the vector can occur.
* **Phosphatase Treatment**: To prevent unwanted re-ligation, vectors are sometimes treated with phosphatase to remove phosphate groups, ensuring that ligation only occurs with the insert present.
* **Gateway Cloning Advantages**:
  + Provides a precise and efficient method for DNA manipulation.
  + Reduces the likelihood of incorrect insert orientation.
  + Minimizes the occurrence of false positives in cloning experiments.

Next Gen Sequencing Videos

* **Introduction**: Revolutionizing personalized medicine, genetic diseases, clinical diagnostics; high throughput, simultaneous sequencing of multiple individuals.
* **Common Features**:
  + **Sample Preparation**: Requires a library, obtained by amplification or ligation with custom adapter sequences.
  + **Sequencing Machines**: Amplification on a solid surface; each cluster acts as an individual sequencing reaction.
  + **Data Output**: Provides raw DNA sequences from each cluster.
* **Methods**:
  + **Pyrosequencing**: Monitors nucleotide incorporation via pyrophosphate release, generating light, detected by a camera.
  + **Sequencing by Synthesis (Illumina)**: Uses reversibly fluorescent terminated nucleotides, reading fluorescent signals at each cluster.
  + **Sequencing by Ligation**: Uses 16 8-mer oligonucleotide probes for hybridization and ligation.
  + **Ion Semiconductor Sequencing**: Detects hydrogen ions released during nucleotide incorporation using a semiconductor transistor.
* **Applications and Efficiency**: Offers extensive coverage, capable of sequencing multiple genomes efficiently (e.g., 15 individuals in 3.5 days by Illumina HiSeq).
* **Service Availability**: abmgood.com offers various NGS services including whole genome sequencing, exome sequencing, RNA sequencing, etc.
* **Sanger Sequencing**
  + **Introduction**: Developed in the 1900s, gold standard for DNA sequencing.
  + **Process**:
    - Uses high fidelity DNA-dependent polymerase, a single primer, and deoxynucleotides.
    - Mix includes four di-deoxynucleotides, each with a unique fluorescent dye, preventing further DNA extension when incorporated.
    - Electrophoresis separates DNA molecules by size, sequence read via fluorescent emission.
  + **Current Use**: Routine sequencing applications, validation of NGS data.
  + **Technology**: Modern instruments use automated capillary electrophoresis.
* **General Information**
  + **Coverage Requirement**: Minimum of 30x coverage for useful whole genome sequencing data.
  + **Comparative Efficiency**: Different technologies vary in output and suitability for specific genomes (e.g., only pyrosequencing suitable for E. coli genome at required coverage).
  + **Web Access**: Detailed service and technology descriptions, pricing, and bioinformatics solutions available on the company's website.
* **DNA Sequencing Importance**
  + **DNA Structure**: Composed of four nucleotide bases (A, G, C, T).
  + **Purpose**: Allows scientists to read genetic information, leading to significant scientific and medical advancements.
* **Historical Progression**
  + **Early Methods**: From Sanger sequencing to more advanced systems like Applied Biosystems and Genome Analyzer.
  + **Technological Evolution**: Continuous search for more accurate and efficient sequencing technologies.
* **Next Generation Sequencing (NGS)**
  + **Revolutionary Impact**: Fundamentally changed scientific research, making sequencing faster and more cost-effective.
  + **Cost Efficiency**: Current costs of NGS are significantly lower than any previous DNA sequencing technologies.
  + **Key Player**: Illumina, a major biotechnology company, significantly contributes to NGS advancements.
* **Illumina's NGS Process**
  + **Library Preparation**:
    - Fragment DNA samples.
    - Attach adapters to both ends of DNA fragments.
    - Perform simultaneous fragmentation and adapter ligation.
    - Amplify using Polymerase Chain Reaction (PCR) and purify through gel electrophoresis.
  + **Cluster Generation**:
    - Load DNA library into a flow cell.
    - Attach DNA fragments to flow cell’s surface.
    - Perform bridge amplification to ensure each DNA fragment is properly amplified.
  + **Sequencing**:
    - Add fluorescently labeled nucleotides.
    - Utilize Illumina’s technology to detect nucleotide incorporation in real-time based on fluorescence.
  + **Data Analysis**:
    - Visualize and align sequenced DNA with a reference genome.
    - Use bioinformatics software to identify variances and analyze data.
    - Employ computational methods for further analysis and visual model generation (e.g., phenetic trees).
* **Scientific and Clinical Impact**
  + **Broad Applications**: Aids various scientific fields by allowing quick and efficient processing of millions of DNA sequences.
  + **Era of Advancements**: Marks the beginning of a new era in scientific research with its high-throughput capabilities and low-cost efficiency.

Crispr/Cas9 Videos

**CRISPR Overview**

* **Origin**: Identified in E. coli; acronym stands for Clustered Regularly Interspaced Short Palindromic Repeats.
* **Structure**:
  + **Repeats**: Short DNA segments (20-40 letters), palindromic.
  + **Spacer DNA**: Unique segments between repeats; originally puzzled scientists.
* **Function**:
  + Acts as a bacterial immune system against viruses (e.g., bacteriophages).
  + Captures viral DNA in spacers for future immunity.

**CRISPR-Cas System**

* **Associated Genes**: CRISPR-associated (cas) genes produce cas proteins.
* **Proteins**:
  + Helicases: Unwind DNA.
  + Nucleases: Cut DNA.
* **Process**:
  + Transcription and translation of cas proteins and CRISPR RNA.
  + CRISPR RNA guides cas proteins to viral DNA to disrupt it.

**CRISPR-Cas9 Specifics**

* **Discovery**: By Jennifer Doudna and Emmanuelle Charpentier.
* **Organism**: Streptococcus pyogenes.
* **Key Component**: Cas9 protein, a dual nuclease.
* **RNA**:
  + CRISPR RNA: Fits into Cas9.
  + Tracer RNA: Stabilizes CRISPR RNA; combined into a guide RNA (gRNA) chimera.

**CRISPR Applications**

* **Gene Editing**:
  + Disables target genes by cutting DNA, followed by cell repair leading to mutations or gene inactivation.
  + Can insert new genes by introducing a template DNA during repair.
* **Potential Uses**:
  + Cure genetic diseases (e.g., cystic fibrosis).
  + Engineer new traits in organisms, including humans.

**CRISPR's Impact**

* **Simplification**: Simplifies the genetic editing process by using gRNA to direct Cas9.
* **Versatility**: Capable of editing DNA at multiple sites simultaneously.
* **Ethical Considerations**: Opens possibilities for extensive genetic modifications in living organisms.

**Overview of DNA and Genes**

* **Genome Composition**: Each cell has a copy of our genome, consisting of over 20,000 genes and 3 billion DNA letters.
* **DNA Structure**: Double helix, with base pairing rules where adenine (A) pairs with thymine (T), and guanine (G) with cytosine (C).
* **Gene Function**: Shapes individual and species characteristics, impacts health, and influences disease risk.

**Advances in Gene Editing**

* **Gene Control**: Essential for understanding gene function; historically difficult to achieve in living cells.
* **CRISPR Introduction**: A breakthrough method allowing precise DNA editing, based on a natural bacterial defense system against viruses.

**CRISPR Mechanism**

* **Components**:
  + **Cas9**: A nuclease enzyme that cuts DNA.
  + **Guide RNA**: Matches DNA sequence of target, directs Cas9.
  + **PAM Sequence**: Recognition site on DNA where Cas9 binds and unzips DNA to check for target match.
* **Process**:
  + When guide RNA matches target DNA, Cas9 cuts the DNA.
  + Cell's repair mechanism, often error-prone, leads to mutations that can disable or alter genes.

**Applications and Implications**

* **Precision Editing**: By introducing a desired DNA sequence during the repair, specific gene modifications can be achieved.
* **Research and Medicine**:
  + Enables creation of transgenic animals and cultured cells with targeted mutations.
  + Facilitates studies on complex diseases involving multiple genes.
* **Potential Uses**: Ranging from basic research and drug development to agriculture and potentially treating genetic diseases in humans.

**Advantages of CRISPR**

* **Efficiency**: Capable of targeting multiple genes simultaneously.
* **Versatility**: Applicable in various biological and medical sciences.

**Discovery of CRISPR**

* **Origin**: Noticed in bacterial chromosomes as sequences of repeats and spacers derived from viruses.
* **Early Observations**: Associated with genes encoding proteins similar to those involved in DNA repair.
* **Hypothesis**: Proposed as a bacterial immune system, acquiring and using viral sequences to defend against infections.

**Research Progression**

* **2000s**: Confirmed that CRISPR systems are bacterial adaptive immune systems.
* **Mechanism**:
  + Foreign DNA from viruses is integrated into CRISPR loci.
  + Transcribed into CRISPR RNA (crRNA).
  + Forms complexes with Cas proteins to target and neutralize viral DNA.

**Collaboration and Development**

* **Collaboration**: Met Emmanuelle Charpentier; focused on the CRISPR system in *Streptococcus pyogenes*.
* **Key Discovery**: Cas9 protein's role as a dual-RNA-guided DNA endonuclease, capable of precisely cutting DNA.

**CRISPR-Cas9 System**

* **System Components**: Guide RNA, tracrRNA, and Cas9 protein.
* **Functionality**: RNA molecules guide Cas9 to specific DNA sequences to induce cuts.
* **Simplification**: Engineered a single guide RNA to simplify the system, making it programmable for targeted DNA cleavage.

**Experimental Validation**

* **Method**: Used guide RNAs to direct Cas9 to specific sites on a DNA plasmid, demonstrated by gel electrophoresis.
* **Significance**: Validated the programmability of Cas9, allowing for precise genome editing.

**Applications and Implications**

* **Genome Editing**: CRISPR-Cas9 enables targeted gene editing by inducing double-stranded DNA breaks, repaired via non-homologous end joining or homology-directed repair.
* **Biological Research**: Facilitates the study of gene function and genetic diseases.
* **Biotechnology and Agriculture**: Potential applications in modifying plants and fungi for industrial uses.
* **Biomedicine**: Promising tool for developing new therapies for genetic diseases.

**Future Prospects**

* **Expansion**: Rapid adoption and creative applications of CRISPR technology in both academic and commercial settings.
* **Potential**: Broad and unforeseen applications likely to emerge, driven by ongoing innovations in genome engineering.

SiRNA videos

**Overview of RNA Interference (RNAi)**

* **Function**: RNAi is a natural mechanism used by many organisms to control gene expression and can be harnessed as a laboratory tool or potential therapy.
* **Key Components**: Small interfering RNAs (siRNAs) and microRNAs (miRNAs) are crucial types of RNA molecules involved in RNAi.

**Gene Expression and RNAi Mechanism**

* **Gene Transcription**: In eukaryotic cells, genes encoding proteins are transcribed into primary RNA transcripts, which are processed into mature messenger RNA (mRNA).
* **Translation**: mRNA is exported to the cytoplasm where ribosomes translate it into proteins.

**Role of Small RNAs in RNAi**

* **Types of Small RNAs**:
  + **siRNAs**: Derived from longer double-stranded RNAs, either produced internally or experimentally introduced to silence genes.
  + **miRNAs**: Originate from RNAs transcribed in the nucleus, processed into double-stranded precursors, and exported to the cytoplasm.
* **Processing by Dicer**: Both siRNAs and miRNAs are cut by the enzyme Dicer into short, roughly 21-nucleotide segments.

**Formation and Function of the RISC**

* **RISC Assembly**: Short double-stranded RNAs bind to an argonaut protein, forming the RNA-induced silencing complex (RISC) with one RNA strand (guide strand).
* **Function of RISC**:
  + **siRNA**: Directs RISC to bind to specific mRNAs based on perfect complementary base pairing, leading to mRNA cleavage and degradation.
  + **miRNA**: Guides RISC to target mRNAs using partial base pairing via the miRNA seed region, leading to mRNA degradation or inhibition of translation.

**Biological and Research Implications**

* **Regulatory Role**: Small RNAs regulate gene expression by targeting mRNAs for silencing, playing a role in controlling protein production.
* **Applications**: RNAi is instrumental in research for gene function studies and holds potential for therapeutic applications targeting specific genes.
* **Presence Across Species**: Argonaut proteins and their associated small RNAs are found in various organisms including plants, animals, fungi, and some bacteria, highlighting their widespread importance in biological processes.

**Overview of siRNA**

* **Function**: siRNA is primarily an anti-viral defense mechanism in cells that prevents viral RNA from integrating into the cell's genetic material.
* **Action**: Described metaphorically as a "genome ninja," siRNA locates and cuts double-stranded RNA to prevent harm to the cell.

**Utilization in Research and Therapy**

* **Gene Silencing**: Molecular biologists exploit siRNA's natural process to induce gene silencing effects in the laboratory, which has significant implications for gene therapy and research.
* **Central Dogma of Protein Synthesis**: Gene expression follows DNA to mRNA to protein. siRNA targets the RNA stage to prevent translation into protein.

**Mechanism of Gene Silencing via siRNA**

* **Production and Introduction**: In the lab, a double-stranded RNA complementary to the target mRNA is synthesized and introduced into the cell.
* **Cellular Processing**:
  + **DICER Action**: The anti-viral enzyme DICER recognizes the double-stranded RNA as foreign, cutting it into smaller fragments.
  + **Formation of RISC**: These fragments then associate with DICER to form the RNA Induced Silencing Complex (RISC).
* **Activation of RISC**: RISC discards the sense strand and retains the anti-sense strand to locate and bind to complementary mRNA sequences.
* **mRNA Cleavage**: RISC cleaves the targeted mRNA, which is then rapidly degraded, preventing the synthesis of the corresponding protein.

**Advantages of Using siRNA**

* **Ethical and Practical Considerations**: Targeting RNA (versus DNA) avoids the ethical and technical complexities associated with permanent genetic modifications.
* **Effectiveness**: The process effectively silences genes without altering the underlying DNA sequence.

**Mechanism of RNAi**

* **siRNA and miRNA**: These small RNA molecules play a crucial role in gene regulation by interfering with the expression at the mRNA level.
* **Gene Silencing Process**:
  + Double-stranded RNA is introduced into the cell.
  + Dicer enzyme cuts the RNA into smaller pieces.
  + These pieces form the RNA-Induced Silencing Complex (RISC).
  + RISC uses one strand of RNA to locate and cleave complementary mRNA, preventing translation into protein.

**Applications of RNAi**

* **Research and Therapy**: RNAi's role in gene therapy is highlighted as a powerful tool for artificially regulating gene expression without altering the DNA itself.
* **Practical Demonstration**: The process shows how targeting mRNA with RNAi can effectively silence specific genes.

**Ethical and Practical Considerations**

* **Preference for RNAi**: Due to ethical concerns around genetic engineering, RNAi is preferred as it does not involve altering the DNA directly.
* **Versatility and Safety**: RNAi is presented as a safe and versatile method for gene regulation, particularly in complex cellular environments.

Lecture Notes

Genetic Engineering/DNA Manipulation

* Goal: The aim is to insert and express a gene (a piece of DNA) in a model organism or cell to study its function.
  + Allows expression in transgenic organisms/ models, as mutations
* Key Terms
  + mRNA (messenger RNA): A type of RNA that carries instructions from DNA to make proteins.
  + cDNA (complementary DNA): A form of DNA made from mRNA, useful for studying genes.
  + ORF – open reading frame: length of RNA/ DNA that can be translated into protein
    - Start and stop codon and triplets of nucleotides
  + Coding sequence- part of the ORF that codes for protein
  + PCR (Polymerase Chain Reaction): A method to make many copies of a specific DNA segment.
  + Plasmid: A small circular piece of DNA used to insert genes into cells.
  + Restriction Digest: A process that cuts DNA at specific spots to study or modify it.
    - Preparative – to insert something
    - Diagnostic – looking for something
  + DNA Ligation: Joining pieces of DNA together.
  + Recombination-Based Cloning: A method to insert a gene into a plasmid or another piece of DNA using enzymes.
  + \*\*Example\*\*: Fluorescent proteins like GFP (green fluorescent protein) are often attached to other proteins to study them.

A diagram of a diagram

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Gene knocks down and knock out

* Knock down = certain protein is overexpressed = downregulate it
* Knock out = completely take out gene entirely

SiRNA= interfere with the RNA to known down the gene

New technology: Deep Sequencing

* DNA sequencing with increased depth
* Depth = higher coverage of sequenced material
  + Same DNA strand sequenced multiple times
* Rapid and more accurate coverage of sequencing

Analyzing Gene Expression

* Goal: To measure how much a gene is "turned on" (expressed) and how much of its corresponding mRNA is made.
  + quantitatively assess the expression of single or multiple genes (transcriptome)​
* Methods
* Northern Blot: A technique to detect specific RNA molecules in a sample.
* RT-qPCR (Reverse Transcriptase Quantitative PCR): Converts RNA back into DNA and then measures how much of it there is to determine gene expression.
* Microarray & RNAseq: These methods measure the expression of many or all genes at once. RNAseq is more detailed and requires computational analysis.

ChIP (Chromatin Immunoprecipitation)

* Goal: To understand how proteins interact with DNA, such as which DNA segments proteins bind to.
* ChIP-on-ChIP and ChIPseq: These methods help identify which sections of DNA are bound by specific proteins, such as transcription factors, across the entire genome.

Protein Analysis

* Goal: To study proteins, their interactions, and their levels inside cells.
* Methods
  + Protein Extraction: Collect proteins from cells or tissues, then break open cells and isolate the proteins.
  + Western Blot: This method checks whether a specific protein is present in a sample and how much is there using antibodies (proteins that recognize other proteins).
  + SDS-PAGE: Separates proteins by size to analyze them.
  + ELISA (Enzyme-Linked Immunosorbent Assay): A method to detect specific proteins using antibodies, good for studying known proteins in many samples.
  + Immunoprecipitation: A way to "pull out" a specific protein from a mixture to study it, often used to understand protein interactions.

Pulse-Chase Experiments

* Goal: To study the lifespan or stability of a protein.
* How it Works: You "label" proteins for a short time (pulse) and then follow what happens to them over time (chase), allowing you to see how long proteins stick around or degrade.

Subcellular Fractionation

* Goal: To study where in a cell different proteins are located (nucleus, membrane, etc.).
* How it Works: You break cells apart and separate their components (like the nucleus, mitochondria, etc.) to see where a protein resides.

Laboratory Diagnosis of Genetic Diseases

* + - * Karyotype and Chromosomal Banding: These methods look at chromosomes (large structures containing DNA) to find abnormalities that might cause genetic diseases.
      * Deep Sequencing: A new method that sequences (reads) DNA much more deeply to find even small errors in DNA more accurately.

Lecture 6

Monday October 28, 2024

A diagram of a protein purification process

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