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A New Phase for WNK Kinase Signaling Complexes as Biomolecular Condensates

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The purpose of this review is to highlight transformative advances that have been made in the field of biomolecular condensates, with special emphasis on condensate material properties, physiology, and kinases, using the With-No-Lysine (WNK) kinases as a prototypical example. To convey how WNK kinases illustrate important concepts for biomolecular condensates, we start with a brief history, focus on defining features of biomolecular condensates, and delve into some examples of how condensates are implicated in cellular physiology (and pathophysiology). We then highlight how WNK kinases, through the action of "WNK droplets" that ubiquitously regulate intracellular volume and kidney-specific "WNK bodies" that are implicated in distal tubule salt reabsorption and potassium homeostasis, exemplify many of the defining features of condensates. Finally, this review addresses the controversies within this emerging field and questions to address.

biomolecular condensates; phase separation; WNK bodies; WNK droplets; WNK kinases

A Brief History

Traditional textbook illustrations of the cell highlight membrane-bound organelles, such as mitochondria, endosomes, lysosomes, nucleus, Golgi, and endoplasmic reticulum. This simplified description simultaneously implies that the remaining cytoplasm and nucleoplasm lack compartmental organization. Therefore, in the absence of membrane enclosures, how do diffuse macromolecules concentrate within the cytoplasm or nucleoplasm to optimize function? There is now a growing appreciation that molecules can organize into functional structures that lack a surrounding membrane, termed "membraneless organelles" (MLOs) (1–3) (commonly used terms are defined in Table 1).

For almost two centuries researchers have observed membraneless compartments in cells and hypothesized about their formation and organization. In the 1830s scientists utilized bright-field microscopy to formally document the first membraneless organelle, the nucleolus (4, 5). Then at the turn of the twentieth century, E. B. Wilson (6) conceptualized the idea of liquidlike phase transitions in biology when describing the protoplasm of the starfish oocyte as a "a mixture of liquids, in the form of a fine emulsion consisting of a continuous substance in which are suspended drops." Further work by Bungenberg de Jong and Kruyt in 1929 (7) published some of the first microscopic images of dense liquid droplets. They coined the terms "unmixing" and "coacervates" to describe the self-assembly of colloids into large liquidlike structures, and these terms were expanded to describe droplets within a cell. During the same time, the Oparin–Haldane hypothesis proposed that electrostatically driven phase separation (complex coacervation) drove the formation of the first protocells that appeared in the Earth's early oceans at the dawn of evolution (8, 9). However, the importance of these biophysical phenomena within the broader context of cellular physiology remained unclear and largely unstudied.

Scientific interest in membraneless organelles was reignited in 2009 when Brangwynne, Hyman, and colleagues (1) discovered that P granules, membraneless organelles obtained from *Caenorhabditis elegans* germ cells, exhibit classic liquidlike behavior including dripping, wetting, and relaxing into spherical structures upon fusion or shearing. Soon after, the Rosen laboratory affirmed that purified protein and RNA can form liquidlike droplets through liquid-liquid phase separation through weak multivalent interactions (2). These discoveries have ushered in an era of phase separation research probing how and why these membraneless complexes form in a variety of synthetic and biological contexts.

In practice, phase separation (PS) is often described by the analogy of a vinaigrette (10). When conditions

Table 1. ***Commonly used words***

Commonly Used Word	Definition
Aggregates	Condensates can devolve into a pathological state with insoluble nonnative interactions, as seen in neurological diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease.
Biomolecular condensates	Membraneless organelles that compartmentalize and concentrate macromolecules, commonly through phase separation.
Complex coacervation	A type of phase separation that was first described over a century ago, occurring in solutions of oppositely charged macromolecules, such as proteins, polymers, and colloids.
Condensatopathies	A dysfunctional condensate that drives a disease phenotype.
c-mods	The development of novel therapeutics that target condensates.
Fluorescence recovery after photobleaching (FRAP)	A microscopy technique to determine the kinetics of diffusion through a cell, using a laser to permanently photobleach a region of fluorescently tagged proteins and then measuring the time it takes for unbleached fluorescently tagged proteins to translocate into the bleached region. This technique can be used to approximate the material properties of a condensate.
Intrinsically disordered region (IDR)	Region in proteins that lacks a stable 3-dimensional structure and instead has a region of disorder that behaves without a predictable structure.
Liquid, gel, solid	Biomolecular condensates can have a continuum of material properties: liquids contain macromolecules that are highly mobile, gels have intermediate mobility, and solids are immobile.
Liquid-liquid phase separation	A type of phase separation involving condensed macromolecules that behave with simple liquidlike properties, forming spherical droplets that fuse, drip, and rearrange rapidly.
Low-complexity domain (LCD)	Region in proteins that lacks the complexity of typical proteins and is made up of only a few amino acids.
Macromolecular crowding	The intracellular concentration of hundreds of thousands of macromolecules creates a crowded environment that can alter thermodynamics and condensate formation.
Maturing or aging	A time-dependent change in the material properties of a condensate from a dynamic state to a less dynamic gel or solid/aggregate.
Membraneless organelle (MLO)	Distinct compartments within a cell that are not enclosed by a lipid membrane.
Multivalent	Macromolecules that contain $>$1 binding site capable of reversible low-affinity interactions with binding partners.
Percolation and phase separation coupled to percolation (PSCP)	The assembly of membraneless organelles with material properties beyond liquids, such as a gel or solid. Percolation theory describes the process of adding connections or nodes to a network, allowing small, disconnected clusters to cross a percolation threshold and emerge as larger connected networks.
Phase separation (PS)	A biophysical principle that defines the formation of membraneless condensates when a homogeneous mixture transitions into 2 distinct phases. They can have a range of material properties including liquid, gel, and solid.
Prionlike domain	A typical low-complexity domain that is involved in liquid-liquid phase separation.
Scaffoldlike and clientlike	Terms to describe macromolecules within a condensate, where scaffolds can self-associate and drive phase separation, whereas clients partition into the scaffolds.
Stickers-and-spacers model	A concept adapted from the theory of associative polymers describing the reversibility of phase behavior, with stickers being motifs that drive noncovalent cross-linked interactions and spacers are motifs that impart flexibility and promote solvation.

are thermodynamically favorable, a mixed solution of oil-vinegar will spontaneously demix into two phases, with a dense phase containing oil-oil (i.e., macromolecules) and a diluted phase of vinegar-vinegar (i.e., solvent) (11, 12). In this example, demixing occurs because of hydrophobic forces; however, within a cell the forces and factors that drive phase separation are more complex and can generate a continuum of material properties that are just beginning to be resolved. Initial descriptions of phase-separated complexes involved simple liquidlike properties, forming spherical droplets through the process of liquid-liquid phase separation (1, 2). However, many membraneless organelles are not conventional liquids and instead contain material properties more akin to a gel or a solid with complex viscous and elastic properties that are governed by time (FIGURE 1). For these membraneless organelles, demixing does not occur through simple liquid-liquid phase separation but through a process termed "phase separation coupled to percolation" (PSCP) (13, 14). This term has been adapted from soft-matter physics and percolation theory to describe molecular phase transitions that arise via the merging of small, disconnected clusters into large, reversible cross-linked networks to generate membraneless organelles with gellike properties (15).

As the field of membraneless organelles continues to grow and our understanding deepens, the terms used to define their assembly and material properties will continue to evolve. In 2017, the term "biomolecular condensate" was adopted to provide a broad unifying term for these mesoscale membraneless compartments that concentrate biological molecules (3). In recent years, there has been exponential growth in the field (FIGURE 2), bringing together a variety of disciplines including polymer chemistry, soft-matter physics, biochemistry, biology, bioengineering, physiology, medicine, and pharmaceuticals. As the field of biomolecular condensates expands, it is becoming clear that these structures are evolutionarily conserved across all kingdoms of life, suggesting their importance in calibrating cellular efficiency and survival.

The Forces and Factors Driving Phase Separation

In this section we review the forces and factors that influence condensate formation including *1*) macromolecule sequence and structure, *2*) condensate composition and material properties, *3*) cellular environment and stressors, and *4*) condensate disassembly processes.

Sequence and Structure

A fundamental property of biomolecular condensates is that they contain multivalent molecules, with multiple binding sites capable of simultaneous reversible low-affinity interactions with binding partners, similar to LEGOs assembling and disassembling through interlocking connections (FIGURE 3). Studies have revealed that increasing valency decreases the

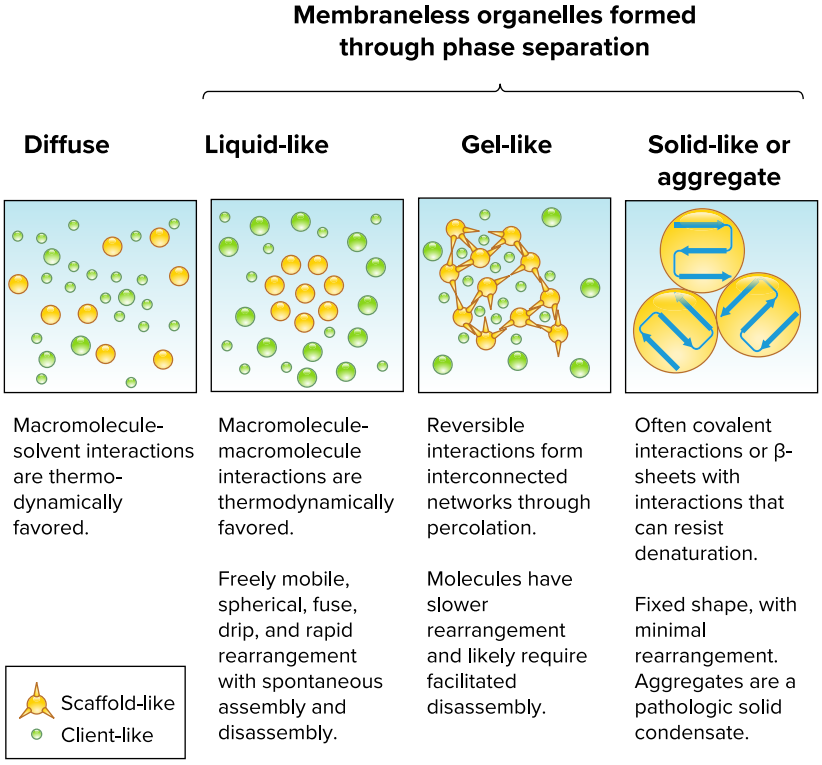

FIGURE 1. **The continuum of material properties of biological condensates, including liquidlike droplets, gellike, and solidlike**.

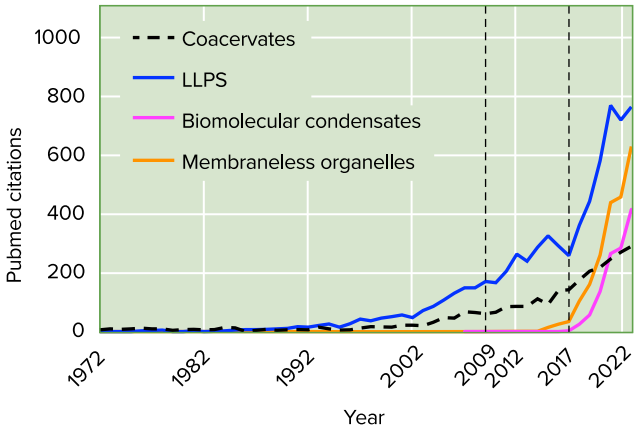

FIGURE 2. **The emergence of terminology to describe biological membraneless organelles**
The emergence of terminology to describe biological membraneless organelles that form within the cytoplasm and nucleoplasm and the exponential growth reflects the growing interest in the field. In 1929, Bungenberg de Jong et al. (7) established the term "coacervate" to describe the self-assembly of colloids into large liquidlike structures. In 2009, Brangwynne et al. (1) adopted the term "liquid-liquid phase separation" (LLPS) to describe liquidlike membraneless compartments in germline P granules. In 2017, Banani et al. (3) coined the term "biomolecular condensates" to describe any membraneless organelle that concentrates molecules to drive cellular organization.

concentration required for condensate formation (2). The formation of biomolecular condensates is also governed by intrinsically disordered regions (IDRs) and low-complexity domains (LCDs) within the protein sequence. Intrinsically disordered regions have been described as "wobbly bits of protein" lacking structure (16) as they do not adopt a predictable three-dimensional (3-D) folded structure and instead they have a range of conformations (17). Despite the absence of a distinct structure, the sequence and length help to govern reaction specificity and the material properties of the biomolecular condensate. Although intrinsically disordered regions were once thought to be rare, bioinformatics studies indicate that up to 50% of all proteins and >70% of signaling proteins in the eukaryotic genome contain intrinsically disordered regions (18).

The intrinsically disordered regions typically contain low-complexity domains (LCDs) where a few amino acids are overrepresented (19, 20). Generally, low-complexity domains are thought to promote condensate formation; however, there are examples of low-complexity domains increasing protein solubility to prevent or modify condensate formation (21). A framework for understanding how low-complexity domains can both drive and suppress condensate formation is the "stickers-and-spacers" model (22–24). This model was adapted from the theory of associative polymers, which describes the reversible phase behavior of polymers (25). Within this model "stickers" are motifs that drive intra- and intermolecular noncovalent cross-links such as hydrogen bonds, ionic interactions, and cation-π or π-π interactions. "Spacers" are interspersed residues that impart flexibility and determine solubility. Engineering specific mutations to the low-complexity domains has revealed that the number and patterning of the stickers and spacers within the sequences alter the formation of biomolecular condensates, and mutations to stickers have a stronger effect than changes to spacers (26).

Classic examples of LCDs are prionlike domains (19, 20). Prions are proteinaceous infectious agents implicated in the formation of self-propagating amyloid aggregates causing deadly spongiform encephalopathies (27). Prionlike (noninfectious) aggregates have been linked to age-related neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and Huntington's disease (28). However, more recent studies suggest that prionlike domains do not always lead to irreversible aggregates but rather can facilitate reversible biomolecular condensate formation and play a role in normal cellular physiology. In addition to prionlike domains, alternative domains have been demonstrated to drive the assembly of biomolecular condensates. These include non-prionlike intrinsically disordered regions (17), hydrophobic domains (29–31), coiled-coil domains (32), and RNA binding domains containing arginine/glycine-rich (RGG) domains (33). Factors that determine whether a domain promotes solubility versus pathological aggregation include time, abundance, concentration, and disease-associated mutations (20).

Condensate Composition and Material Properties

Just as a single instrument does not produce a symphony, a single constituent within a condensate cannot impart all function; rather it is the composition and interaction of molecules within the condensate that drive their activity and material properties. Each biomolecular condensate can contain up to hundreds of different types of molecules (3, 34). Many of these molecules are dispensable for phase separation, and there are examples where only one or a limited number of key components are required for condensate formation, as in the

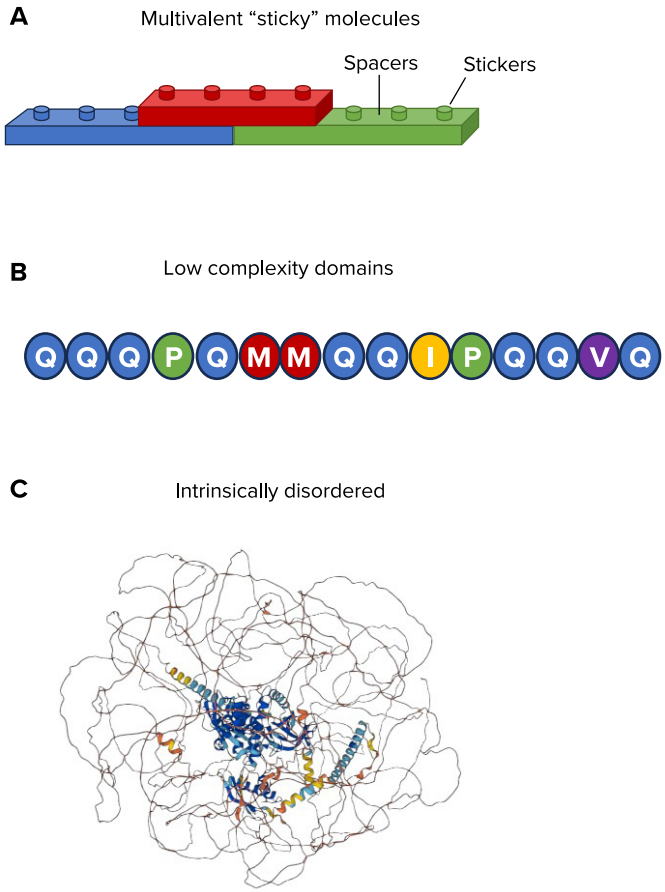

FIGURE 3. **Sequence and structure driving phase separation**
A: multivalency describes multiple regions within a molecule that are capable of weak interactions with binding partners. Regions containing “sticky” motifs can drive noncovalent cross-linked interactions, and these sticker regions are interspersed with spacer regions that impart flexibility and promote solvation. A simple illustration is LEGO building blocks that are composed of studs interspersed between flat spaces that facilitate the reversible binding of blocks. *B*: protein sequences that promote phase separation often contain low-complexity domains, which are regions that lack diversity in amino acids. For example, the sequence shown here is from condensate-prone region of the *Drosophila melanogaster* WNK kinase COOH-terminal domain and is enriched in glutamines. *C*: intrinsically disordered regions that lack 3-dimensional structure are important for phase separation. This is the alpha-fold predicted structure of human WNK1 protein based on the amino acid sequence (UniProt Q9H4A3). The NH_2-terminal kinase domain is predicted to have a 3-dimensional structure; however, the alpha-fold program is unable to predict a structure for the long COOH-terminal tail.

case of Kidney-Specific With-No-Lysine (KS)-WNK1, which is required for WNK body formation (discussed below) (35). The essential molecule(s) are defined as “scaffoldlike,” and the other proteins and nucleic acids within the biomolecular condensate are termed “clientlike” depending on the degree to which deletion of the factor affects the formation of the biomolecular condensate (36, 37). Scaffoldlike molecules can self-associate and drive phase separation, whereas clientlike molecules partition into the scaffolds.

The interactions between scaffoldlike and clientlike molecules can alter the material properties of condensates, which encompass dynamic liquid droplets, reversible gels, and arrested solids (FIGURE 1). One way to assess the material properties of condensates is to utilize a microscopy technique termed fluorescence recovery after photobleaching (FRAP) to monitor molecular diffusion (38). Photobleaching of a fluorescent tag is an irreversible process, so for a region to regain fluorescence an unbleached tagged protein must move into that region. When FRAP is performed on a liquidlike condensate, molecules within the condensed phase exhibit rapid fluorescence recovery as fluorescently tagged molecules easily exchange into and out of the bleached condensates (39, 40). The interactions within a liquidlike condensate tend to be weak multivalent interactions that are easily formed and broken. A liquidlike condensate has a rearrangeable shape capable of forming spheres, fusing, dripping, and conforming to its surroundings within seconds (1, 10). Examples of membraneless organelles that exhibit liquidlike behavior in vivo include P granules (1), P bodies (37), and WNK droplets (40).

Solidlike condensates have a fixed shape often maintained via chemical covalent bonds and strong physical bonds such as those seen in β-strands (25). These bonds are stable until enzymatic disaggregation, or they can be irreversible and resistant to denaturation (41–43). During FRAP experiments, solidlike condensates exhibit delayed and incomplete recovery

(39, 41, 42). Our preference is to refer to pathological examples of solid biomolecular condensates as "aggregates," as they generally contain molecules that are in a nonnative dysfunctional state with irreversible interactions (44). Examples of pathological aggregation in biology include the plaques seen in Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease (28). Importantly, not all solid condensates are pathological, and examples of functional solidlike condensates include Balbiani bodies (41), A bodies (43), prionlike CPEB proteins involved in long-term memory (28), and prionlike signalosomes in the innate immune response (45).

Condensates can also adopt an intermediate state between liquid and solid that is more akin to a gel. Gels resist transient deformation but with time and compression can slowly rearrange and change shape. In FRAP experiments gels tend to have delayed recovery similar to a solid, reflecting short-term immobility of the complexes. Examples of gellike structures include centrosomes (32), the nuclear pore complex (46), RNA repeats (47), and the inner core of stress granules (48). Moreover, the physical state of a condensate can change with time and stress, known as maturation or aging (3, 44). Condensates can be multilayered and can transition between material states. Examples of multilayered condensates include the nucleolus and stress granules, which both have inner gellike cores with surrounding shells with liquidlike properties (48, 49).

The Cellular Environment and Stress

The internal cellular environment often hovers at or near metastability, so that slight deviations to the environment can result in rapid intracellular responses toward either dissolution or condensation (50, 51). Thus, numerous examples have emerged where phase separation constitutes a fundamental mechanism by which cells sense and respond to stress. Examples of environmental variables that influence condensate formation include macromolecular crowding, temperature, energy availability, and factors that influence charge-based interactions.

In a cell the concentration of macromolecules (i.e., proteins and nucleic acids) is between 150 and 400 mg/mL, with up to 40% of the intracellular volume composed of macromolecules (52–56). This creates an extremely crowded environment that entropically favors molecular associations that are on the brink of spontaneous aggregation (20, 57); it would be almost impossible to maintain this concentration within a test tube without it precipitating out of solution (58). To defend against catastrophic overcrowding, cells spend a substantial amount of energy tightly regulating the concentration of macromolecules. In vivo, cells utilize natural crowding agents such as ribosomes to tune molecular crowding. Delarue and colleagues (57) found that mammalian target of rapamycin complex (mTORC)1 promotes ribosome production and that ribosome abundance is directly correlated to phase separation. For more information on the topic of molecular crowding and its relationship to phase separation please refer to the reviews in Refs. 52, 59–61.

Temperature is another environmental factor that induces condensate formation. For example, in the plant *Arabidopsis thaliana*, raising the temperature to 34°C (93.2°F) induces the formation of a type of biomolecular condensate called stress granules, which are thought to promote cell survival (62). Agriculturists are investigating how to leverage temperature-induced condensates to develop more thermotolerant crops, notably in the context of global warming and diminishing crop yields (63, 64). Other physiological stressors that alter phase separation include nucleic acid abundance (33), energy availability (ATP) (58), intracellular pH (21), and inorganic salts (65). All these factors can alter charge-based electrostatic interactions that drive the formation of condensates. For example, ATP at physiological millimolar concentrations can prevent condensate formation and solubilize biomolecular condensates through ionic interactions mediated by the negatively charged triphosphate groups (58, 66, 67). Thus, environmental factors and physiological stressors such as molecular crowding, temperature, energy availability, and ionic strength can modify the propensity to form and tune biomolecular condensates.

Condensate Disassembly

An emerging question within the field is, What regulates spontaneous versus facilitated disassembly of condensates and reentry into a diffuse phase? It has been proposed that spontaneous reversal may facilitate rapid reentry, whereas slower disassembly may facilitate a timed or graded response (51). Spontaneous disassembly classically occurs with liquidlike droplets; when the stimuli or stress is removed the macromolecules reenter into the diffuse state. A more complex problem, and one that is critical for human disease, is the facilitated disassembly of gels and solids.

An important model for the study of condensate disassembly has been cytoplasmic ribonucleoprotein (RNP) granules (i.e., stress granules). The dissolution of stress granules often requires energy-consuming mechanisms, with increases in ATP promoting a more diffuse state whereas ATP depletion promotes a more gellike state (47, 56, 58). ATP-driven machinery such as molecular motors, helicases, and chaperones regulates the fluidity of stress granules and potentially condensates as a whole (48, 68–71). Chaperones that promote reentry of stress granules into a diffuse state include several heat shock proteins (Hsp104, Hsp110,

Hsp70, and Hsp40) (21, 43, 48, 70, 72–75) and chaperone-like nuclear-import factors (76). Although chaperone-mediated dissolution of stress granules constitutes a key pathway for disassembly, aberrant stress granules that are resistant to disassembly can be transported along microtubules to aggresomes for eventual autophagy-mediated degradation (72, 75).

Another mechanism for the disassembly of condensates includes posttranslational modifications that act as rapid and reversible mechanisms to regulate phase separation (77). Posttranslational modifications that neutralize a charged amino acid, such as acetylation (78), tend to inhibit phase separation, whereas phosphorylation (79–81), which adds a negatively charged phosphate, can either promote or inhibit phase separation by inducing electrostatic attraction or repulsion. Kinases can regulate their cycling between condensates and cytosol through phosphorylation. For example, the dephosphorylation of a kinase by phosphatases can drive the formation of condensates, and the autophosphorylation of a kinase can promote reentry into the cytosol. This dynamic cycling has been observed for the apoptosis signal-regulating kinase 3 (ASK3; MAP3K15) (82) as well as for the dual-specificity tyrosine-phosphorylation-regulated kinase 3 (DYRK3) (81). A similar mechanism has been proposed for ubiquitylation, with polyubiquitylated proteins being shuttled out of the condensate by ubiquilins and targeted for proteosomal degradation (31). Thus, posttranslational modifications have an integral role in modulating the disassembly of condensates.

Condensate Physiology and Pathophysiology

Function of Biomolecular Condensates

Within a cell, biomolecular condensates can create their own microenvironment to facilitate a diverse range of functions. As summarized by Shin and Brangwynne (39), these include concentrating molecules to act as a reaction crucible; alternatively, they can sequester molecules to prevent interactions or they can form organizing hubs, as is the case with centrosomes that form membraneless microtubule organizing centers (32).

Thus, it may seem intuitive that condensates must be physiologically relevant, but demonstrating this in complex model systems has been challenging and has ignited controversy within the field, as highlighted by reviews in Refs. 16, 83–87 and discussed below. Consequently, it has become a priority to shift from engineered in vitro test tube experiments into in vivo living systems, including cellular models (40, 88), plants (89), fungi (90), insects (40), and even rodent animal models (35, 91). These in vivo studies are beginning to support the idea that condensates play a key role in physiology including nerve architecture and plasticity (88, 92), the immune response (79), cell division and growth (32, 93), environmental sensors to promote cellular fitness (29, 73, 89, 90), memory formation (28), and electrolyte homeostasis (91). For additional information about the physiological function of biomolecular condensates please refer to the reviews in Refs. 34, 39, 44, 85, 94.

Role in Disease

Aberrant biomolecular condensates that are clearly linked to diseases have been termed “condensatopathies” (50). Researchers have been particularly interested in how dysfunctional RNA processing condensates (i.e., stress granules) form disease-associated aggregates (95). This pathological transition from stress granule to aggregate has been implicated in many different diseases, and by studying the proteome of mammalian stress granules, Jain et al. (48) were able to link 75 stress granule components to 153 Mendelian diseases including neurological diseases, muscular diseases, and cardiovascular diseases.

Examples of two classic stress granule proteins that have been identified in neurodegenerative diseases are TAR DNA binding protein 43 kDa (TDP-43) and Fused in Sarcoma (FUS). Both are involved in the overlapping syndromes of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (96, 97). Other triggers for neurodegenerative condensatopathies include repeat expansion disorders such as Huntington’s disease and expanded polyQ tracts in spinocerebellar ataxias (47), amyloid formations in Alzheimer’s disease (98), and α-synuclein in Parkinson’s disease and Lewy body dementia (99). Importantly, all these neurological diseases, including ALS and FTD, are diseases of aging. Condensates are prone to aggregate with time because aging favors protein misfolding, amyloid-like interactions, and dysfunctional disassembly (3, 44, 97). However, other condensate-associated neurological diseases can occur in the young, as in the case of *SYNGAP1*-related intellectual disability. SynGAP is a synaptic GTPase-activating protein that is essential for synaptic plasticity and normal brain development. Mutations to the COOH-terminal domain of SynGAP trigger defective condensate formation and neuronal hypersensitivity to stimulation characterized by epilepsy, intellectual disability, and autistic-like features (88, 92).

Dysregulated condensate formation is also associated with cancer. Over 40 cancer-linked proteins have been found in condensates and can cause anomalous condensate assembly through several different mechanisms as reviewed in Ref. 100. It has been predicted that over half of all fusion oncoproteins drive aberrant condensates and alter cell signaling and gene expression (101). For example, atypical liver cancers arise because of the generation of protein kinase A (PKA)

fusion oncoproteins that prevent the assembly of condensates leading to cell proliferation and transformation (102), and hematologic malignancies commonly contain NUP98 fusion oncoproteins that form nuclear condensates that alter gene expression (103).

Therapeutic Targets

Researchers and pharmaceutical companies are eager to target biomolecular condensates to develop newer and better drugs, most notably in cancer and neurodegenerative diseases. The term "condensate-modifying therapeutics (c-mods)" has been coined to classify drugs that target condensates to prevent or reverse disease (50). The current c-mod drug strategies include the elimination of toxic condensates, rebalancing condensate composition, concentrating drugs into condensates, and altering posttranslational modifications (16, 100). A recent advance has been the development of small molecules that can target intrinsically disordered regions, a sequence that has traditionally been considered undruggable because of a lack of structure (104).

In cancer therapeutics, studies have shown that both cisplatin and tamoxifen are antineoplastic agents that partition into condensates (105). Cisplatin has been used since the 1970s to treat a broad range of cancers and acts by cross-linking DNA and inhibiting replication and transcription. The pharmacological activity of cisplatin is enhanced by increasing its concentration up to 600-fold in transcriptional condensates enriched in DNA and increasing the efficacy of DNA platination (105). Likewise, tamoxifen concentrates into transcriptional condensates that contain its protein target, the estrogen receptor. When tamoxifen enters the condensate it forces the estrogen receptor out, hindering condensate formation and inhibiting cell proliferation in breast cancer cells (105). A common issue with both cisplatin and tamoxifen is intrinsic or acquired drug resistance. Recently, researchers have been able to alter tamoxifen sensitivity and resistance through changes to condensate formation, ushering in a new era of condensate therapeutics (105).

A New Phase for WNK Kinases

Up to this point, this review has broadly expounded on biomolecular condensates to establish a framework that can be used to examine With-No-Lysine (WNK) kinases, a family of serine/threonine kinases that undergo phase separation and regulate ion transport. First, we start with the background on WNK1 kinases, describe their sequence and structure, dissect the composition of WNK1-dependent condensates, and study the cellular environment that induces condensate formation and factors that may influence its disassembly. A key takeaway is the introduction of specific terminology for WNK condensates, specifically WNK droplets and WNK bodies (Table 2). Finally, we place WNK1 condensates within a physiological context in both health and disease.

Introduction to WNK Kinases

The WNK1 serine/threonine protein kinase was first cloned from a rat brain cDNA library in 2000 by Cobb and colleagues (106) and was named "With-No-Lysine" because of the absence of its catalytic lysine from its classic location; instead, the lysine is positioned in a different subdomain within the kinase domain. The unusual location of the lysine places it close to a structurally resolved binding pocket for chloride. Chloride binding to this site inhibits WNK1 autophosphorylation and activation (107). WNK1 is the prototypical member of the WNK kinase family, which are all defined by the uniquely placed lysine and chloride-binding domain. WNK homologs have been predicted or identified in protists to humans (but absent from *Saccharomyces cerevisiae*) (40, 108). At least 11 members of the WNK family have been reported in the plant *Arabidopsis thaliana* and are important for plant tolerance to salt, drought, and temperature stress as well as circadian rhythm (109). There is a

Table 2. ***Comparing WNK droplets to WNK bodies***

WNK Droplet	WNK Body
Requires the expression of L-WNK1 COOH terminus	Requires the expression of KS-WNK1 exon 4a
Ubiquitously expressed throughout cells and seen in invertebrates	Exclusively expressed in vertebrate kidneys, specifically the distal convoluted tubule
Stimulated by osmotic stress	Stimulated by potassium deprivation
Contains WNK1, WNK3, and SPAK/OSR1 and promotes the phosphorylation of the WNK/SPAK pathway and its downstream target NKCC1 and KCC.	Contains WNK1, WNK4, and SPAK/OSR1 and promotes the phosphorylation of the WNK/SPAK pathway, and presumably its downstream target NCC.
Electron microscopy hyperdense membraneless organelle	Electron microscopy hypodense membraneless organelle
Liquidlike material properties based on microscopy and FRAP	Gellike material properties based on microscopy and FRAP

FRAP, fluorescence recovery after photobleaching; KCC, potassium-chloride cotransporter; NKCC1, sodium-potassium-chloride cotransporter 1.

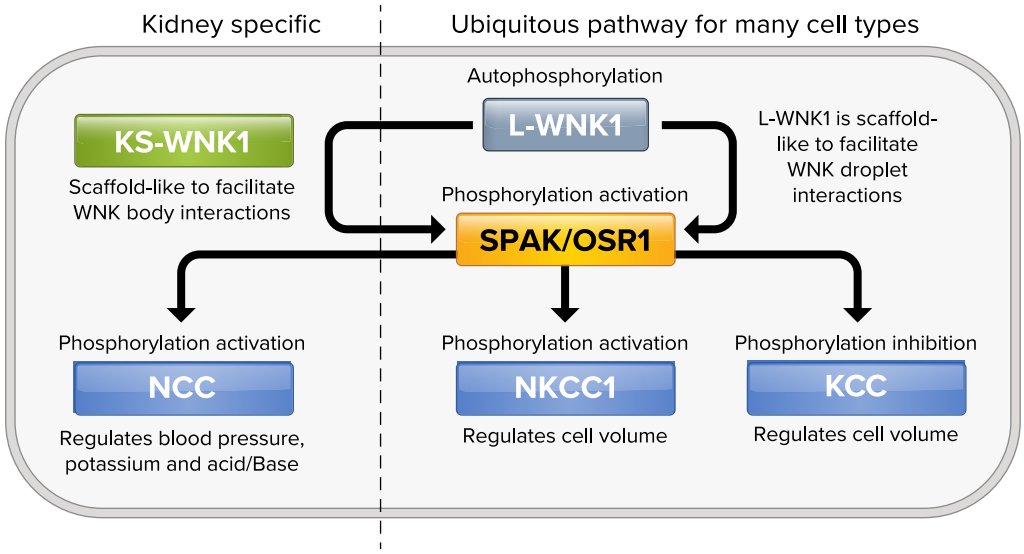

FIGURE 4. **Schematic for the WNK1-SPAK/OSR1 kinase pathway and its downstream activation of electroneutral cation-chloride transporters within the SLC12A family**
KCC, potassium-chloride cotransporter; NCC, sodium-chloride cotransporter; NKCC1, sodium-potassium-chloride cotransporter 1.

single homolog present in *Drosophilia melanogaster* and *Caenorhabditis elegans*, whereas mammals have four WNK kinases (WNK1–4).

Shortly after WNK1 was cloned, interest in these kinases increased when gain-of-function mutations were identified as underlying the rare Mendelian condition of familial hyperkalemic hypertension (FHHt) (also known as Gordon syndrome or pseudohypoaldosteronism type II) (110). This disorder features increased renal sodium reabsorption and impaired potassium and hydrogen excretion resulting in hypertension, hyperkalemia, and metabolic acidosis. Before this discovery, it had long been known that people with this disease could be treated with hydrochlorothiazide, an inhibitor of the sodium-chloride cotransporter (NCC) in the kidney's distal convoluted tubule (111). This observation linked WNK kinases to the phosphorylation of NCC in the kidney and eventually to other electroneutral cation-chloride transporters within the SLC12A family including the sodium-potassium-chloride cotransporter (NKCC1) and the potassium-chloride cotransporter (KCC). Through a series of studies, it was revealed that autophosphorylation of WNK1 and WNK4 kinases phosphorylates STE20/SPS1-related proline/alanine-rich kinase (SPAK) and its close homolog Oxidative stress response kinase-1 (OSR1) to initiate the kinase cascade that phosphorylates and either activates NCC/NKCC1 or inactivates KCC (112–114) (FIGURE 4). From cellular models to whole animals, the WNK/SPAK/OSR1 kinase cascade has been shown to be important for volume and electrolyte regulation.

L-WNK1 and WNK Droplets

The human WNK1 gene encodes ~2,382 amino acids within 28 exons generating a very large protein (250 kDa) with multiple isoforms and splice variants (108) (FIGURE 5*A*). The two major isoforms are the kinase-active Long (L)-WNK1 and the truncated kinase-deficient Kidney-Specific (KS)-WNK1. L-WNK1 is expressed ubiquitously throughout the body, with highest expression in testis, heart, lung, kidney, placenta, skeletal muscle, and brain (115). L-WNK1 contains an NH_2-terminal kinase domain that requires autophosphorylation of Ser382 for activation (112). The kinase domain is directly followed by an RFXV-binding PASK/FRAY homology 2 (PF2)-like autoinhibitory domain, which suppresses WNK1 kinase activity (116). The COOH terminus is extremely long and intrinsically disordered, with low-complexity and prionlike domains. The COOH terminus also contains coiled-coil domains and SPAK/OSR1 binding motifs (FIGURE 5*B*). Given these features, it is perhaps not surprising that this large COOH-terminal intrinsically disordered region drives the formation of WNK biomolecular condensates during exposure to hypertonicity-induced molecular crowding (40, 117). These condensates are liquidlike, as they fuse, wet against surfaces, are electron dense, and contain dynamic material that recovers quickly in FRAP studies (FIGURE 6). Thus, we refer to them as "WNK droplets."

The first publication identifying L-WNK1 suggested that it functions as an osmosensor that increases its kinase activity in response to hyperosmotic stress (106). L-WNK1 autophosphorylation is induced in a dose-dependent manner by treating cells with either sorbitol, NaCl, or KCl (106, 112). Later studies demonstrated that intracellular chloride depletion also stimulated L-WNK1 autophosphorylation (113). Groundbreaking work in 2007 by Zagórska and colleagues (112) revealed that hyperosmotic stress rapidly and reversibly redistributes L-WNK1 to intracellular punctate structures, which were thought to be "intracellular vesicles." They reported that under normal conditions L-WNK1 is diffuse throughout the cytosol but upon hyperosmotic

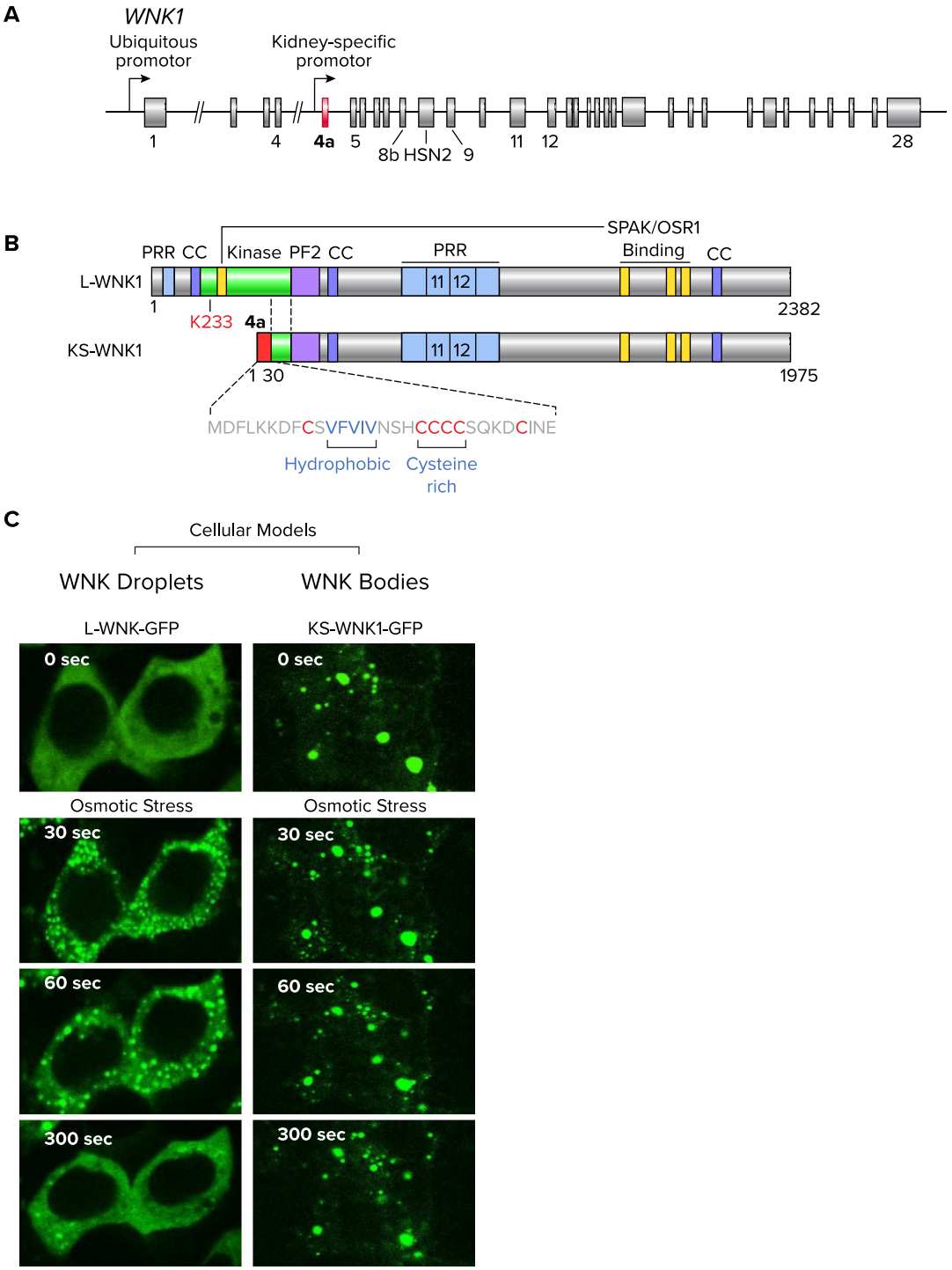

FIGURE 5. **WNK**

A: mammalian WNK1 contains 28 exons, with alternative isoforms including KS-WNK1 and WNK1/HSNS. *B*: comparing the structures of L-WNK1 and KS-WNK1. KS-WNK1 expression is regulated by an alternative promoter that produces a protein lacking exons 1–4 and instead contains a unique exon 4a. This exon contains a unique cysteine-rich domain required for scaffold formation. From exon 5 and beyond, KS-WNK1 is identical to L-WNK1. *A* and *B* adapted from Boyd-Shiwarski et al. (35), with permission from *Molecular Biology of the Cell*. *C*: the effect of osmotic stress on WNK droplets and WNK bodies (previously unpublished images). HEK293 cells were transiently transfected with either L-WNK1-green fluorescent protein (GFP) or KS-WNK1-GFP and then treated with sorbitol (50 mM). Within seconds, L-WNK1 formed liquidlike droplets that dissipated after 300 s, whereas KS-WNK1 had an adynamic response to the same osmotic stress.

stress it is driven into highly mobile cytoplasmic structures and this relocalization is dependent upon the L-WNK1 COOH terminus. At the time, the authors hypothesized that these structures were recycling endosomes because of a lack of alternative explanations to describe their results. Their observations were ahead of the science, as 2 years later Brangwynne and colleagues (1) first described the phenomenon of

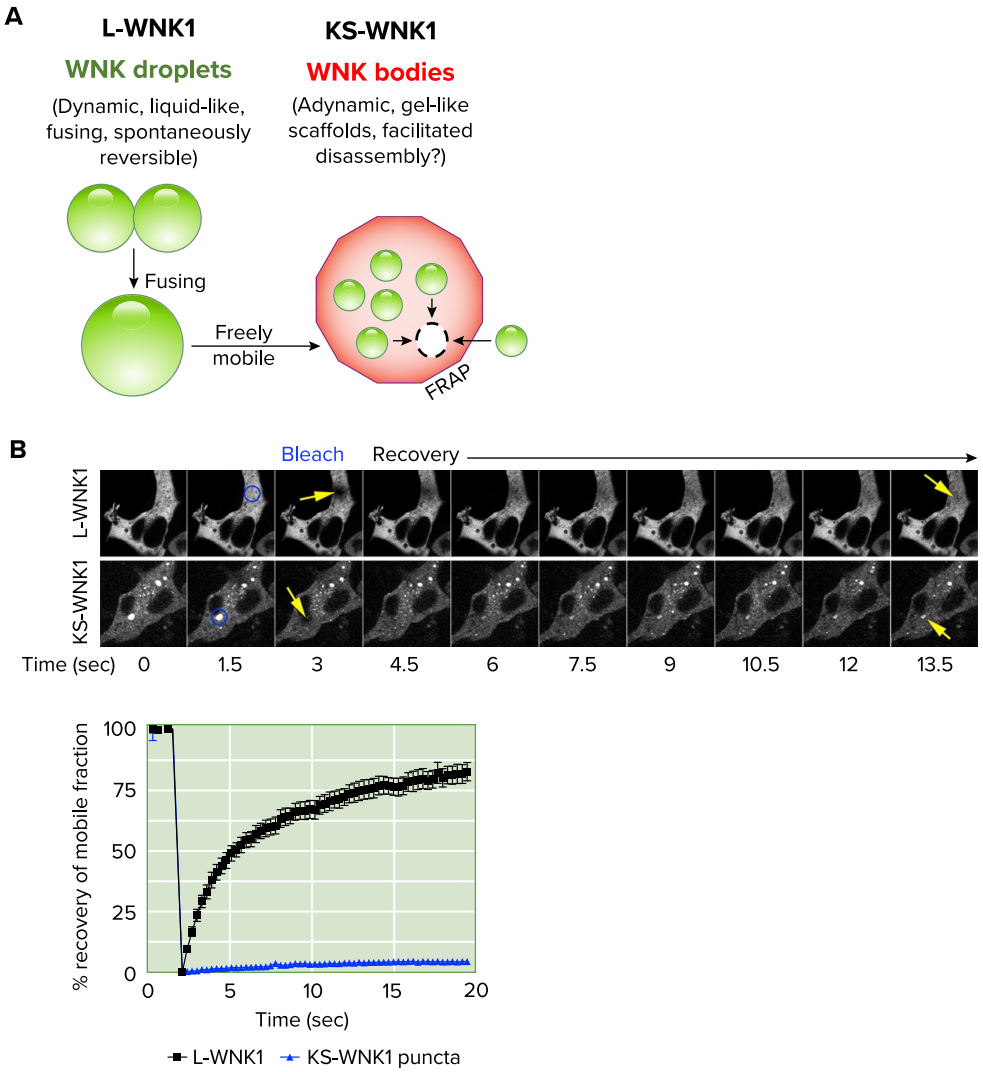

FIGURE 6. **Material properties of WNK droplets and WNK bodies**
A: cartoon illustration of the material properties of WNK droplets and WNK bodies and their response to fluorescence recovery after photobleaching (FRAP) experiments. *B*: actual FRAP experiments performed with HEK293 cells transiently transfected with either L-WNK1-green fluorescent protein (GFP) or KS-WNK1-GFP. Yellow arrows indicate the photobleached region. L-WNK1 had a rapid recovery, with >75% recovery by 20 s, vs. KS-WNK1, which had a delayed recovery, with <1% by 20 s. Image adapted from Ref. 35, with permission from *Molecular Biology of the Cell*.

cellular liquid-liquid phase separation, and it would be another 15 years before these L-WNK1 puncta were shown to be membraneless liquidlike WNK droplets, not membrane-bound endosomes (40).

Within WNK droplets, L-WNK1 has scaffoldlike behavior, colocalizing with other WNK kinases, most notably WNK3. The downstream kinases SPAK/OSR function as clients, as they require L-WNK1 (and possibly WNK3) to enter the condensed phase. Once inside the condensate, SPAK/OSR1 undergo condensate-dependent activation. They then leave WNK droplets to phosphorylate the SLC12 cotransporters NKCC1 and KCC at the plasma membrane. Phosphorylation of the transporters results in a net influx of sodium, potassium, and chloride, resulting in intracellular volume recovery. A series of experiments in cells revealed that the driver for L-WNK1 phase separation during hyperosmotic stress is macromolecular crowding, supporting a role for L-WNK1 as a molecular crowding sensor. Thus, WNK droplets are L-WNK1-dependent liquidlike condensates that sense molecular crowding and regulate intracellular volume.

KS-WNK1 and WNK Bodies

KS-WNK1 is unique in that it is exclusively expressed in the distal nephron of the kidney. It is derived from an alternative promoter that substitutes the first four exons (437 amino acids) of L-WNK1, including a majority of the kinase domain, with 30 amino acids termed exon 4a (FIGURE 5*B*) (115, 118, 119); thus, KS-WNK1 lacks both kinase activity and the chloride binding domain. From exon 5 onward the COOH terminus of KS-WNK1 is identical to L-WNK1, containing the entire intact COOH-terminal intrinsically disordered region that functions as a driver of phase separation, with presumably similar tendencies toward condensate

formation. Closer inspection of exon 4a reveals that its 30-amino acid sequence is unique and highly conserved across evolution, first emerging in coelacanths, a lobe-finned fish closely related to lungfish. Thus, like many essential genes in the kidney, KS-WNK1 emerged around the time of the terrestrial evolution (35). Exon 4a has both a conserved hydrophobic domain and a cysteine-rich domain, and mutations to either domain prevent the formation of WNK biomolecular condensates (35). KS-WNK1-dependent condensates are different from WNK droplets, as they are electron hypodense, are minimally mobile, retain their shape, and have minimal recovery in FRAP studies (FIGURE 6) (35). They are only expressed in the distal convoluted tubule of the kidney, the same site where NCC is expressed. Given their unique properties that distinguish them from WNK droplets, we refer to them as "WNK bodies."

WNK bodies were first noted as large punctate structures in mouse kidney distal convoluted tubule during hypokalemia induced through either genetic manipulation or dietary deprivation (120–123) (FIGURE 7). These puncta stained positive for proteins within the WNK/SPAK/OSR1 kinase cascade including SPAK/OSR1, phosphorylated SPAK/OSR1, and WNK4, yet at the time it was not clear whether these puncta were artifacts, aggregates, or functionally relevant. Then in 2018, a breakthrough occurred with the discovery that these puncta required the expression of the scaffold-like protein KS-WNK1; thus, they were termed WNK bodies (35). Electron microscopy images of WNK bodies revealed that they were membraneless structures and electron hypodense (35). This hypodense porous structure may be important for infiltration by other macromolecules and could create a mesh that restricts access to larger molecules (93, 125–127). These observations established the strict definition of WNK bodies as KS-WNK1-dependent membraneless condensates that appear in the kidney distal convoluted tubule during potassium stress.

Since WNK bodies are scaffoldlike for proteins in the WNK signaling pathway, it seems likely that they are required for NCC activation, though this has not been formally tested. WNK bodies have been identified in both mouse and human kidneys (35, 128, 129). In mice they appear within 12 h of potassium deprivation and continue to increase in abundance and size throughout the stress. Once the potassium stress is removed, the WNK bodies dissipate within 24–48 h (129, 130). Thus, compared to WNK droplets, which form within seconds and dissolve within minutes, the appearance and dissolution of kidney tubule WNK bodies occur on a much longer timescale.

Many factors about WNK body disassembly remain a mystery, including the exact time course of disassembly and whether chaperones or posttranslational modification is required. Interestingly, when KS-WNK1 is exogenously expressed in HEK293 cells, WNK bodies are constitutively observed, regardless of the extracellular potassium concentration (35), suggesting that this cell model lacks the ability to disassemble WNK bodies. In 2020, Louis-Dit-Picard and colleagues (131) were studying subjects with a mild form of FHHt with hyperkalemic hyperchloremic acidosis without hypertension and identified a novel human mutation in WNK1 that affects

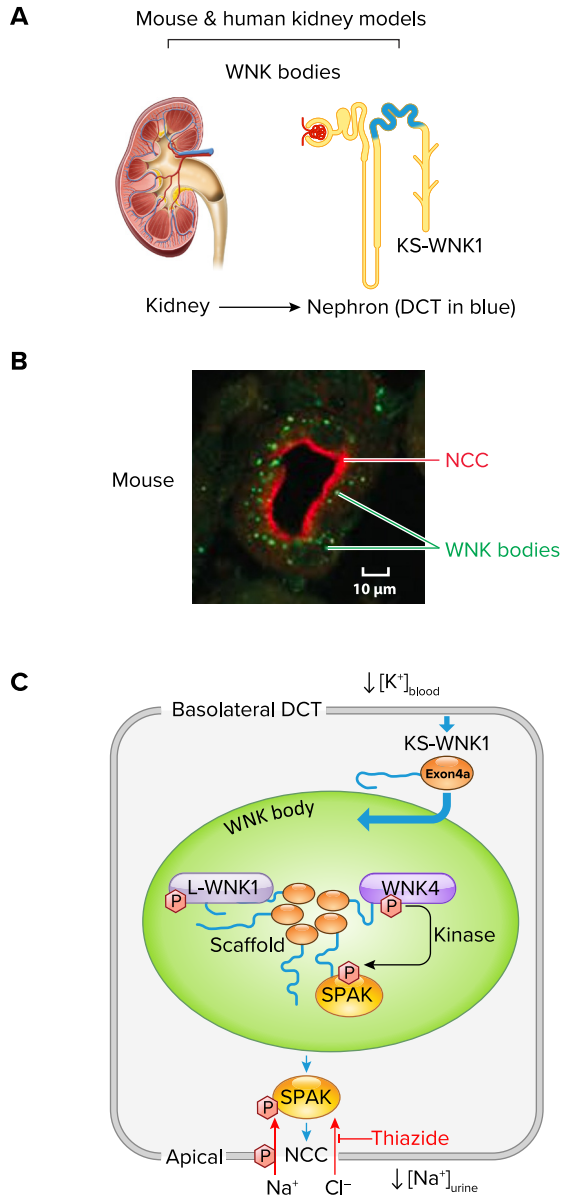

FIGURE 7. **KS-WNK1 is exclusively expressed in vertebrate kidneys**
A: KS-WNK1 is exclusively expressed in vertebrate kidneys and enriched in the distal convoluted tubule (DCT) of the nephron. KS-WNK1 represents 99% of the WNK1 mRNA in the DCT and is 80-fold more abundant than L-WNK1 (124). *B*: images of DCTs obtained from mouse. In mice, immunofluorescence was performed and the sodium-chloride cotransporter (NCC) is shown in red and represents the apical membrane, whereas WNK1 is shown in green and represents WNK bodies (adapted from Boyd-Shiwarski et al. (35), with permission from *Molecular Biology of the Cell*]. *C*: cartoon illustrating how hypokalemia is sensed by the DCT and stimulates KS-WNK1 to form scaffolds that bind the WNK-SPAK pathway, ultimately increasing the phosphorylation activation of the thiazide-sensitive NCC.

binding of the Kelch-like 3/Cullin-3 (KLHL3/CUL3) E3-ubiquitin ligase complex. Gain-of-function missense mutations were identified in WNK1 exon 7 within the acidic motif, a 10-amino acid sequence critical for binding of an E3-ubiquitin ligase complex. These mutations preferentially decreased the ubiquitination and degradation of the KS-WNK1 isoform, rather than L-WNK1, hinting that this complex may be important for WNK body disassembly. In a mouse model engineered to express the human mutation, KS-WNK1 was resistant to ubiquitin-mediated degradation, resulting in enlargement of WNK bodies and inappropriate activation of the WNK-SPAK/OSR1 kinase cascade despite elevated blood potassium levels (131). This led to overactivation of the sodium-chloride cotransporter (NCC) and hyperkalemic metabolic acidosis that corrected with inhibition of NCC with a thiazide diuretic (131).

Interestingly, it has also been reported that KS-WNK1 knockout (KO) mice can exhibit mild overactivation of NCC at baseline (132, 133). Reconciling how both overexpression of KS-WNK1 and loss of KS-WNK1 can result in activation of NCC is an active area of investigation. Studies have shown that KS-WNK1 function is dependent on dietary potassium, with KS-WNK1 inhibiting NCC during higher-potassium diets and activating NCC during lower-potassium diets (91, 129). How fluctuations in potassium alter KS-WNK1 and WNK body formation to act as both an activator and an inhibitor of NCC remains an open question. WNK bodies are rarely present at baseline in vivo but form during low potassium when the WNK-SPAK pathway becomes activated. After low serum potassium, KS-WNK1 KO mice are unable to form WNK bodies, have diminished activation of the WNK-SPAK kinase cascade and decreased NCC phosphorylation, and develop a Gitelman-like syndrome (91, 129). Thus, during low potassium the KS-WNK1 protein, and seemingly WNK bodies, amplify the WNK-SPAK kinase cascade to allow small changes in potassium to be translated into large changes in NCC regulation. A challenge with studying the function of WNK bodies has been to disaggregate their function from that of KS-WNK1. To delineate the function of WNK bodies, future studies are aimed at developing KS-WNK1 mutants that express full-length KS-WNK1 but are unable to form functional WNK bodies.

Interestingly, two studies have reported the appearance of WNK1- and/or SPAK-positive punctate structures in the distal tubule that appear during high-potassium diet (35, 134). These puncta differ from classic WNK bodies as they occur with high-potassium diet. Morphologically, they are more apically located, have a smaller diameter, and are less abundant (35). Furthermore, the puncta do not appear to contain phosphorylated WNK-SPAK proteins based on the diffuse staining in the distal convoluted tubule when using phospho-specific antibodies (134). Future work must identify what proteins are contained in these structures, whether they are membraneless, whether they facilitate phosphorylation or dephosphorylation or sequester proteins, and what duration and intensity of stress are required for formation. These high-potassium-induced WNK1- and/or SPAK-positive puncta require further characterization before they can be called WNK bodies.

WNKs in Health and Disease

Mutations to WNK1 are classically implicated in the kidney-centric disease of familial hyperkalemia and hypertension, affecting blood volume and electrolyte homeostasis. In humans, numerous WNK1 single-nucleotide polymorphisms (SNPs) have been associated with alterations in blood pressure, salt-sensitive hypertension, and thiazide sensitivity (135–137). It remains to be seen whether the COOH-terminal SNPs alter condensate formation. Beyond the kidneys, WNK1 and its downstream targets are also important for development and other diseases. For example, knocking out the L-WNK1 gene in mice results in embryonic lethality (138), whereas conditional endothelial knockouts have abnormal cardiovascular development and defects in angiogenesis (139).

WNK1 mutations can also cause hereditary sensory and autonomic neuropathy type 2 characterized by an inability to perceive touch, heat, and pain. This disease occurs because of mutations to a specific isoform of the WNK1 kinase termed WNK1/HSN2. This isoform contains the novel exon, HSN2, located between exons 8 and 9 that is exclusively expressed in neuronal tissue and enriched in dorsal root ganglia (FIGURE 5*A*) (140, 141). HSN2-exon mutations lead to the expression of a truncated nonfunctional protein that lacks a majority of the intrinsically disordered COOH terminus. Thus, it presumably alters biomolecular condensate formation, similar to engineered L-WNK1 truncating mutants studied in HEK293 cells (40). Other neurological roles for WNK1 include its potential as a therapeutic target to treat neuropathic pain (142). WNK1/HSN2-mutant mice or pharmacological inhibition of WNK1 diminished hypersensitivity to cold and mechanical stimuli in a neuropathic pain injury model. Moreover, there is growing evidence that WNK/SPAK/OSR1 promotes the pathogenesis of stroke (143). In mouse stroke models, there is a significant increase in brain WNK1 and SPAK/OSR1 24 h after stroke, and pharmacological inhibition of this pathway improves mouse stroke outcomes (144). If these data are confirmed in humans, it is feasible that the WNK/SPAK/OSR1 pathway could be a pharmacological target to mitigate the harmful effects of stroke (145).

It is widely recognized that WNK1 is dysregulated in cancer, promotes cell proliferation, angiogenesis, metastasis, and migration, and is inversely associated with prognosis. It would not be surprising if the WNK1 therapeutic targets for cancer involve biomolecular condensates. Increased expression of WNK1 is seen in

many different cancers including gliomas (146), breast cancer (147, 148), colon cancer (149), and hepatocellular carcinoma (150). A high burden of WNK1 expression is associated with increased breast cancer invasiveness (147) and increased mortality with hepatocellular carcinoma (150). Thus, WNK1 remains a promising target in cancer therapy. For a more detailed description regarding WNKs and cancer please see the review in Ref. 151.

Condensates and Controversies

The exponential growth in the field of condensate biology has led to paradigm shifts in our understanding of basic cellular organization and challenged the classic teachings of cellular biology. The claims that biomolecular condensates have such a prolific, diverse, and fundamental role is not without its controversies, as highlighted in several recent reviews (11, 13, 16, 83–87). These concerns include that the field has come to premature conclusions using in vitro models and more work needs to be done to show in vivo physiological relevance. They also argue that more rigorous characterization is needed, and terminology must be carefully applied. We agree that it is crucial to develop accurate vocabulary for this new field encompassing broad disciplines of biology, polymer chemistry, and biophysics and recognize that there will be debates and compromises in terminology as this field matures.

Questions to Address

Beyond terminology, there are fundamental questions that remain to be addressed regarding biomolecular condensates. Many questions revolve around their basic physiology and functional relevance (Table 3). There is no doubt that these structures can be observed in vitro when highly concentrated under specific conditions, but the droplets could be an artifact of the overexpressed model system (11). What happens in vivo at physiological concentrations, and how do we study phase separation in living cells? One can imagine the challenges of isolating the function of the condensate from the function of diffuse proteins or nucleic acids within a cell. To separate the function of the condensate, the molecule of interest must be expressed within the cell, but the molecule's ability to form condensates must be prevented. Furthermore, because of the nature of condensates, advanced imaging techniques are required to observe them in vivo. Our own studies have shown that endogenous WNK1 can form liquid droplets in cellular models; however, these droplets are smaller than in ectopic overexpression systems, and detection is hindered by the diffraction-limited resolution of light microscopy. It is possible that WNK droplets are occurring during milder stress and at lower WNK1 concentrations that cannot be detected by our assays. This alludes to the question of scale and how many interacting macromolecules are required to meet the definition of a biomolecular condensate (11, 34). With tens to hundreds of molecules within a condensate (3), what are the components within each given condensate, and what are the minimum components required for function? How do the dynamics and interactions of molecules differ within the condensate compared to outside the condensate? Finally, how is specificity within the condensate compartment generated and maintained (127)?

One way to begin to answer these questions is by generating condensate-prone and condensate-resistant chimeras that can be expressed in living cells or by analyzing nature's own chimeras through natural sequence variations. We propose that L-WNK1 and KS-WNK1 are an archetype that can be used to address in vivo questions regarding condensates in both cells and animals. For example, when terrestrial kidneys evolved from coelacanths, the kidneys naturally engineered the WNK1 chimera "KS-WNK1" that contained the condensate-prone COOH terminus with a unique NH_2 terminus that drove the formation of WNK bodies during potassium-depleted states (35). By studying the function of WNK bodies we can begin to understand the role of condensates in animal and human physiology.

Other examples include engineered L-WNK1 mutants that express either the NH_2-terminal domain, the catalytic inactive isoform, or COOH-terminal chimeras. A series of experiments in cells revealed that L-WNK1 requires the COOH-terminal intrinsically disordered domain to drive phase separation in the presence of hyperosmotic stress to activate the WNK/SPAK/OSR1 pathway and rescue cell volume (40). Conversely, a truncated NH_2-terminal construct containing only the first 491 amino acids regulates a different kinase pathway, the mTORC2 signaling pathway. Remarkably, it does not appear that catalytic activity is necessary for L-WNK1's regulation of the mTORC2 signaling pathway (152), and this truncated construct does not undergo phase separation (40). This generates interesting questions about WNK1 catalytic activity and the role of phase separation for distinct signaling pathways.

A different engineered L-WNK1 chimera swapped the COOH-terminal domain required for phase separation with intrinsically disordered low-complexity domains from either FUS or TDP-43 (40). The NH_2-terminal WNK1 1–494 truncated mutant did not undergo phase separation or rescue cell volume. Conversely, both the WNK1 1–494-FUS and 1–494-TDP-43 proteins formed droplets in response to osmotic stress and were able to restore intracellular volume, albeit slightly less efficiently than full-length L-WNK1. This transforms our understanding of how protein sequences predict specificity and function. For L-WNK1 the sequence of the COOH terminus does not appear to define function; rather it is the tendency to undergo liquid-liquid phase separation that confers its ability to activate SPAK and rescue cell volume.

Table 3. ***Questions to be addressed***

Broad Questions for Condensates	How the Question Applies to WNKs
How can a condensate coexist with other condensates within the same cell that have shared molecules but different functions?	How do WNK droplets and WNK bodies interact and scaffold within the same cell, since they have identical COOH termini that bind the WNK-SPAK pathway?
What determines a physiological and pathological assembly? What drives disease conversion, and can we harness this for novel drug discovery?	How do WNK droplets and WNK bodies disassemble? With enough time and stress, can WNK bodies transition into pathological aggregates, or do they remain physiological reversible condensates regardless of environment?
	How do Mendelian variants to the WNK1 gene affect WNK condensate formation?
	How are WNK condensates involved in disease processes including electrolyte handling, volume regulation, neurological diseases, and cancer?
What is the function of condensates in vivo at physiological concentrations, and how do we study phase separation in living cells?	At baseline, or during mild stress, do WNK droplets and WNK bodies occur at the nanoscale level below the detection limit of our current assays? How can we develop new techniques to study them in vivo?
What are the components within each given condensate, and what are the minimum components required for function?	How many WNK-SPAK interactions are required for condensate formation? Does KS-WNK1 scaffoldlike behavior lower the threshold for condensate formation?
How do the dynamics and interactions of molecules differ within the condensate compared to outside of the condensate?	How does the function of L-WNK1 and KS-WNK1 differ within and outside of WNK condensates?
How is multicomponent specificity generated and maintained?	What determines whether L-WNK1 behaves as a scaffoldlike protein or a clientlike protein?
	What is the composition of WNK bodies vs. WNK droplets?
	How do the WNK condensates that appear during high potassium stress differ from WNK bodies?

This theory is strengthened by natural sequence variation within the COOH terminus of WNK kinases. The ability of the COOH terminus to promote phase behavior is conserved throughout evolution despite poor sequence homology. When comparing human WNK1 to *Drosophila melanogaster* WNK or *Caenorhabditis elegans* WNK there is only 22% sequence identity in the COOH-terminal domain. Yet they all contain nearly identical disorder tendency, low sequence complexity, and prionlike domains. Thus, this permissive evolutionary adaptation altered amino acid sequence but preserved phase behavior (40). An emerging concept is that intrinsically disordered regions often have poor sequence alignment yet retain conserved sequence features important for phase separation, since natural selection depends on function, not sequence (94, 153). It should be noted, however, that for some proteins that form condensates an intrinsically disordered region that drives phase separation may also contain features that confer functions outside of its phase behavior. This was recently shown for the ARID1A subunit of the chromatin remodeler cBAF, which contains an intrinsically disordered region that drives phase separation but also mediates partner interactions that are essential for its function (154). Dissecting intrinsically disordered region-mediated phase separation behavior from other encoded functions will require a deeper understanding of how intrinsically disordered sequences mediate specific physiological responses. For now, many questions remain, and new approaches are required to answer these questions. There are several excellent reviews and studies that address methods and limitations for rigorously studying biomolecular condensates (see Refs. 11, 34, 38, 57, 85, 155).

Conclusions

In summary, this review integrates the canonical WNK kinase pathway with condensate biology. We propose that L-WNK1 exists in protists to humans to counter hyperosmotic stress and macromolecular crowding. Macromolecular crowding stimulates L-WNK1 to form WNK droplets through multivalent interactions of the intrinsically disordered COOH-terminal domain. These droplets colocalize with other WNKs and undergo auto-/transphosphorylation. The phosphorylated-activated WNKs mediate the phosphorylation-activation of SPAK/OSR1 within the droplets. Then, through

an unknown mechanism, SPAK/OSR1 leaves the WNK droplet to target the cation-chloride cotransporters in the cell membrane, regulating ion transport and restoring intracellular volume (40) (FIGURE 4).

From this ancient WNK1/SPAK/OSR1 kinase pathway emerges KS-WNK1, a kinase-deficient isoform appearing in land-dwelling vertebrate kidneys to protect against life-threatening potassium depletion. How serum potassium depletion induces WNK body formation remains unknown. Perhaps it causes localized macromolecular crowding (61), or alterations to cellular energy, or changes in transcription/translation. WNK droplets are presumably forming within WNK bodies, leading to a secondary condensed liquidlike phase within the primary condensed gellike phase. This relationship between WNK bodies and WNK droplets can be described as a cellular "field of dreams": if you scaffold it, they will come. In the kidney, we propose that these biomolecular condensates amplify the activation of NCC to diminish downstream urinary sodium delivery. This limits the exchange of sodium for potassium, decreases urinary potassium excretion, and restores potassium homeostasis.

It has become increasingly clear that WNKs are a class of proteins expressed in simple unicellular organisms to complex humans that contain the fundamental elements required for phase separation and the formation of biomolecular condensates with varying material properties. Evolution has generated natural WNK isoforms with cell-specific expression through splice variants, isoforms, and alternative promoters. By studying these in vivo isoforms, including L-WNK1, KS-WNK1, WNK1/HSN2, and the human disease-causing mutants, we can study how and why nature evolved membraneless compartments to organize the cell. Future studies combining new experimental tools with WNK kinases will continue to shed light on how these endogenous condensates form and disassemble in vivo and how the native components within condensates confer function and test the physiological relevance of biomolecular condensates. ■

We apologize to all colleagues whose work could not be cited because the abundance of literature in the field and space limitations.

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