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Review

Galvanization of Protein–Protein Interactions in a Dynamic Zinc Interactome

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The presence of Zn^{2+} at protein–protein interfaces modulates complex function, stability, and introduces structural flexibility/complexity, chemical selectivity, and reversibility driven in a Zn^{2+}-dependent manner. Recent studies have demonstrated that dynamically changing Zn^{2+} affects numerous cellular processes, including protein–protein communication and protein complex assembly. How Zn^{2+}-involved protein–protein interactions (ZPPIs) are formed and dissociate and how their stability and reactivity are driven in a zinc interactome remain poorly understood, mostly due to experimental obstacles. Here, we review recent research advances on the role of Zn^{2+} in the formation of interprotein sites, their architecture, function, and stability. Moreover, we underline the importance of zinc networks in intersystemic communication and highlight bioinformatic and experimental challenges required for the identification and investigation of ZPPIs.

Highlights

Presence of Zn^{2+} at protein–protein interfaces modulates complex stability, protease resistance, introduces structural flexibility, spatial complexity, chemical selectivity, and, above all, reversibility driven in Zn^{2+}-dependent manner.

Zinc homeostasis is strictly regulated at the cellular level; thus ZPPI formation and dissociation is tightly interwoven with Zn^{2+} status. Factors influencing complex stability obey covalent bond formation, allosteric modulation, metal-coupled folding, proton-coupled assembly mechanisms, and post-translational and chemical modifications.

Intersystemic zinc network communication reveals the *terra incognita* of Zn^{2+}-involved protein–protein interactions.

Difficulties in prediction of interprotein zinc sites need to be overcome by functional cellular and biochemical investigation. Combination of labile Zn^{2+}, proteome, and global gene expression investigation supported by mass spectrometry and fluorescence-based studies in numerous cell types will allow new ZPPIs to be identified.

Protein–Protein Interactome and the Role of Metal Ions

A complete picture of protein–protein interactions (PPIs), also known as the **protein–protein interactome** (see Glossary), is crucial to understand cell molecular machinery at the system biology level [1–3]. Identification of protein molecular complexes requires the description of various aspects, such as composition, component **affinity**, and lifetime with series of spatial and physicochemical properties describing the **interface** between proteins. Considering both the affinity and lifetime of proteins to interact, obligate and nonobligate complexes can be found, where the latter are divided into permanent (mostly strong, irreversible) and transient (reversible, frequently with medium affinity). Transient complexes are characterized by smaller interfaces, frequently dependent on specific recognition patterns, where the limited surface meets the requirement of constituents to fold and exist independently without aggregation [4,5]. Their interfaces contain more residues of higher polarity stabilized by salt bridges and hydrogen bonds with more chain turns to gain flexibility [6–8]. The specificity of such interactions is also elevated by the utilization of the same interface for multiple interactions across evolution, where the local environment has been affecting the fast adaptation of mutations [9,10].

Within the widely examined PPI network, there is an additional small component to consider. Besides its almost negligible size compared with macromolecule complex diameter, metal ions have a critical impact on structure, function, and stability of protein complexes. Even though their influence on living organisms has been investigated for decades, the description of the employment of metal ions in PPIs is still vague. How is the cell able to utilize the smallest components available in handling PPIs? Some answers derive from consideration of d-block metal ions' properties such as ionization potentials, ionic radii, or d-shell electronic configurations, which influence the type of protein functional groups that participate in metal binding [11]. The preference of metal ion binding is also driven by the specific spatial arrangement of binding amino acid side groups (**coordination sphere**). Interfaces of metal-involved interactions are characterized by small interface areas and usually fold into stable structures when bound. Such transient PPIs provide a quick response to cellular perturbations and changes during signaling and regulatory processes enabling, for example, messengers to reach the target [12]. It may explain the frequent involvement of metal

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ions such as Ca^{2+} or Zn^{2+} in signaling pathways where the ability to form and break interactions readily, essential to respond quickly, is interconnected with high stability of such interactions when needed [13–15]. The presence of a metal ion at the interface also confers specificity, since the formation of an interprotein complex needs the spatiotemporal orchestration of both metal ions and protein subunits concentration [16]. Moreover, environmental changes are sensed by metal-involved PPI, since the net outcome in complex formation is dependent on the redox potential, pH, post-translational and chemical modifications, competitive ligands, etc. [11,17–19].

Among all biologically essential metal ions, Zn^{2+} interacts specifically with the largest number of proteins (~10% of proteins encoded in the human genome), which defines the human **zinc proteome** [20]. Its multiple functions, investigated for decades, led to the discovery of catalytic and structural zinc sites such as zinc hydrolases and zinc fingers, but the picture of an ion utilized as a source of PPIs has just started to evolve [17,21,22] and the role of Zn^{2+} at protein interfaces has emerged [18,22,23]. What makes it so special in harnessing PPI? In answering that question, one should consider the relative metal abundance and availability under physiological conditions along with metal ion physicochemical properties [24]. Chemically, Zn^{2+} is the only essential metal ion that can switch the ligand geometry without major energetic disfavor [11]. The substantial rise of coordination dynamics is due to the filled d-shell leading to a lack of ligand field stabilization. The utilization of oxygen, nitrogen, and sulfur donors contributes to the modulation of the coordination number, where moderate Lewis character of the Zn^{2+} is demonstrated. Associated geometrical preferences are then elevated, which makes Zn^{2+} especially useful at protein–protein interfaces. Moreover, ligand reactivity is maintained with diverse substitution kinetics of Zn^{2+}, which depends on metal-to-protein affinity and composition of the first and second coordination sphere and is demonstrated by exchange rates from seconds to years [11,17]. It is a known fact that thermodynamically stable Zn^{2+} protein complexes can be kinetically inert and labile, especially when an intermediate ternary complex is formed, accelerating the rate of metal ion dissociation and further transfer [25]. The complex mode of action has to be supported by specific regulation. On the one hand, it results in the particular utility of Zn^{2+} for proteins to assemble, but on the other hand, such an interplay of properties has to be strictly governed by the cell (Box 1). Besides proteins, Zn^{2+} also interacts

Glossary

Affinity: a relationship between a metal ion and its binding site described by the law of mass action.
Allosteric activation: regulation of macromolecule function by binding an effector molecule at a site other than that responsible for native function.
Coordination sphere: inner sphere containing ligands directly interacting with metal ion.
Interface: spatial arrangement between interacting molecules.
Labile Zn^{2+} concentration: the concentration of exchangeable Zn^{2+} complexed by LMWL.
Metal-coupled folding: domain or protein folding initiated and dependent on Zn^{2+} binding.
Protein–protein interactome: complete map of protein interactions that can occur in a living organism.
Zinc interactome: a set of all Zn^{2+} binding species, including zinc proteome and LMWL.
Zinc pool: a set of dynamically exchangeable Zn^{2+} species.
Zinc proteome: a set of all Zn^{2+} protein complexes.
Zn^{2+}-involved protein–protein interactions (ZPPIs): set of protein–protein interactions with the involvement of Zn^{2+}.

Box 1. Properties of Cellular Zinc Pool

The steady-state intracellular labile Zn^{2+} concentration ($[Zn^{2+}]_i$) must be maintained under tight control to prevent miss-metalation. To do so, homeostatic machinery keeps Zn^{2+} within strictly regulated (buffered) concentrations to act upon the Irving-Williams series that ranks the propensity of metal ions to undergo substitution with other ligands. Intracellular availabilities of metal ions are sensed to selectively bind a cognate metal ion to a protein according to free energies for metal complex formation [24,75]. Uncontrolled access to the cellular zinc pool would render many metalloproteins dysfunctional and likely turn the cell into cell death pathways. One way that eukaryotic evolution solved the problem is cellular compartmentalization, where the cellular membrane plays the role of a fence to separate different concentrations of Zn^{2+} with a specific set of zinc transporters as gates for Zn^{2+} to flow (Figure I) [76]. The importance of compartmentalization is manifested in a high concentration of labile Zn^{2+} in vesicles such as insulin-storing granules or zincosomes and placement of Zn^{2+} in intracellular organelles, from where it can be released on demand [77]. In fact, $[Zn^{2+}]_i$ of a eukaryotic cell is buffered in the range from low nanomolar to picomolar, which is several orders of magnitude lower in comparison to total Zn^{2+} of 0.1–0.3 mM [11,13]. A key player in the specialized zinc buffering system is the metallothionein, that can bind up to seven Zn^{2+} with nanomolar to picomolar affinity [78,79]. Moreover, the zinc buffering machine was found to be insufficient to mechanistically describe a steady-state model based on cytosolic zinc proteins to match the time-dependent changes in intracellular labile Zn^{2+} concentration under Zn^{2+} changes (zinc transients). Therefore, the muffling model was introduced as a time-dependent, combined effect of binding and flux, coined initially to describe damping changes in labile intracellular Ca^{2+}, Mg^{2+}, and protons [37]. Muffling reactions involve binding of the surplus of Zn^{2+}, shuttling it into subcellular stores, and/or removing it from the cell, in order to restore the labile Zn^{2+} concentration to its original value and augment the buffering capacity [37,80]. As fluorescent Zn^{2+}-specific probes appeared, subnanomolar concentration values were confirmed in different cell types (erythrocytes, cardiomyocytes, cortical neurons) and distinct cell lines (INS-1, HeLa, HEK293, MCF-7, TamR), summarized in Table S1 (see the supplemental information online). Subcellular compartments such as granules, ER, Golgi, and mitochondria seem to contain lower $[Zn^{2+}]_i$ [81,82]. Evaluation of extracellular labile Zn^{2+} concentration indicates the submicromolar range, being consistent with the affinity of a major storage zinc protein, albumin, and the affinity of zinc transporters [83].

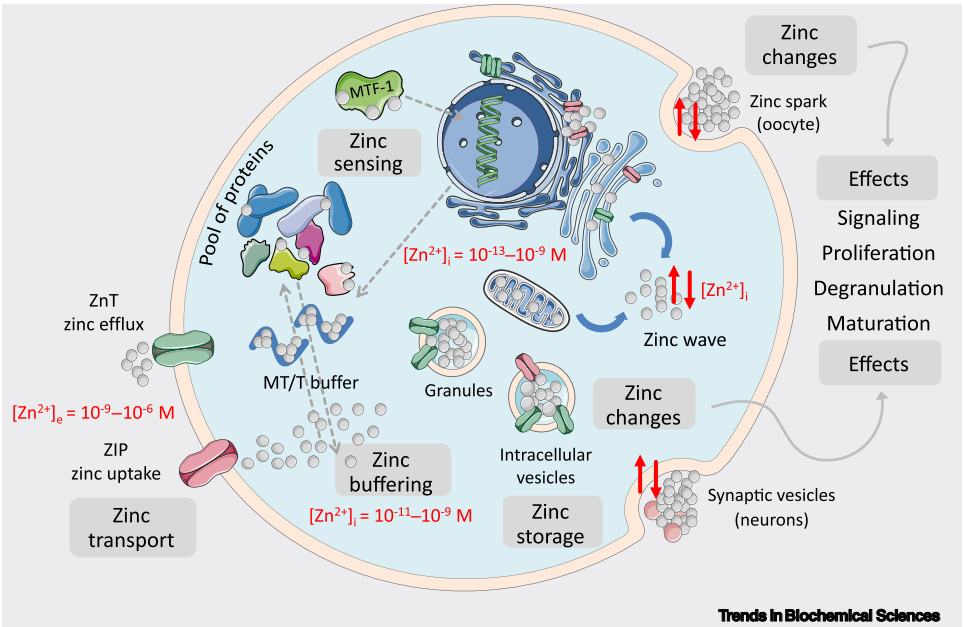

Figure I. Schematic Representation of the Cellular Zinc Pool. Gray arrows and circles demonstrate zinc flow and Zn^{2+} ions for which LMWLs are omitted, respectively. Families of zinc transporters ZnT and ZIP (green and red cylinders) are primarily involved in Zn^{2+} efflux and influx across the cellular membranes. Under physiological conditions, 14 ZIP membrane proteins and ten ZnTs associated with plasma membrane and intracellular compartments facilitate Zn^{2+} mobilization to maintain the homeostatic level of labile intracellular Zn^{2+} ($[Zn^{2+}]_i$). The main contributors to zinc buffering and storage processes are metallothioneins (MT/T system, blue curved line) that redistribute Zn^{2+} to apo-proteins (colorful shapes) and keep labile Zn^{2+} at the right concentration (10^{-11}–10^{-9} M). A central regulator of MTs and ZnT1 genes, MTF-1 transcription factor (green) upregulates their expression via Zn^{2+} sensing. Transient changes in $[Zn^{2+}]_i$, of which the most prominent examples are zinc sparks, zinc waves, and Zn^{2+} changes in a synaptic cleft, contribute to a range of cellular responses (e.g., signaling pathways, proliferation, degranulation, and/or maturation). The figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com. Abbreviations: LMWL, low molecular weight ligand; MTF-1, metal-response transcription factor 1; MT/T, metallothionein/thionein; ZIP, SLC39 zinc transporter family; ZnT, SLC30 zinc transporter family; $[Zn^{2+}]_e$, extracellular labile Zn^{2+} concentration.

with nucleic acids, peptides (including glutathione), amino acids, specific chelators and cargos, vitamins, carboxylic acids, and a vast number of organic compounds that are products/intermediates of metabolism. Integral parts of the interactome are inorganic anions (e.g., P_i, HCO_3^-, HS^-, SO_4^{2-}, Cl^-), which either complete the coordination sphere in larger complexes or outcompete Zn^{2+}, forming low molecular weight ligand (LMWL) complexes [11,26]. Both Zn^{2+} complexes with LMWLs and previously-listed non-protein cellular components are major parts of the kinetically and thermodynamically labile **zinc pool**, which also contains Zn^{2+} complexes of some labile proteins such as metallothioneins (Box 1). Rapidly exchanging zinc pool species function either independently or together with the more kinetically inert zinc proteome, forming ternary complexes required for Zn^{2+} transfer, modulating the function of other molecules, or playing a distinct role in intra- and intercellular signaling [11,13]. The variety of Zn^{2+} interactions with multiple molecules brings us to the definition of the term '**zinc interactome**' as the pool of all Zn^{2+} species, including complexes with macromolecules and LMWL. Due to its heterogeneous composition and kinetic and thermodynamic character, the zinc interactome is a dynamic 'organism' where all components permeate each other.

Taking into account the increasing number of structurally identified and/or experimentally captured Zn^{2+}-involved PPI interactions, we here explore this still unchartered territory and present a comprehensive outlook of the state of knowledge of **Zn^{2+}-involved protein–protein interactions (ZPPIs)** occurring in a dynamic zinc interactome. This includes screening the protein database for functional ZPPIs and the captured Zn^{2+}-involved dynamic network, together with factors governing Zn^{2+}-mediated PPIs.

Screening of Structural Protein Database for Functional ZPPIs

Reasonably good sources of new ZPPIs are deposited protein structures, although, due to applied conditions during crystallization or protein complex preparation, the vast majority of them seem to be artificial. More detailed information regarding ZPPI selection can be found in the supplemental information online. To confirm the functionality of potential ZPPIs, a complementary route of biochemical identification and cellular verification must be performed to avoid misclassification. The literature supplies a range of examples described from multiple perspectives (Box 2). Thus, the comprehensive study of ZPPI architecture and function is needed. Complete systematic attempts to collect Zn^{2+}-binding sites have been considered over the years, where the MetalPDB or ZincBind database were inspected based on different criteria [27,28]. MetalPDB contains all structurally characterized metal binding sites; however, its disadvantage is the fact that the metal binding sites described in it are based on asymmetric units and not on biological assemblies. ZincBind solves this obstacle by utilizing biological assemblies; nevertheless, none of the databases allows for the selection of interprotein metal binding sites. The obstacles encountered led us to analyze the largest database available, Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB), with its account of biological assemblies of

Box 2. Zn^{2+}-Involved PPI Network

Over the years the number of identified interprotein Zn^{2+} complexes has been growing. Examples presented below show their versatility in protein complex composition and zinc site architecture (Figure I) and multiple branches where they should be considered during experimental attempts.

Among transporters and channels, the binuclear zinc site in the bacterial zinc transporter Yiip serves to maintain domain integrity and to allosterically modulate transport kinetics [84]. Zn^{2+} is also needed to form the functional membrane pore of, for example, the tetrameric voltage-gated K_v channel that brings conformational plasticity and membrane excitability [85,86]. Several examples are found in cytokine families such as lactogenic hormones [hGH, human placental lactogen (hPL), and human prolactin (hPRL)], prolactin receptor (hPRLr), interferons, tumor necrosis factor (TNF), and nerve growth factor (NGF) being important in structure and stability maintenance, leading to changes in signal transduction pathways [87–89]. A relevant example here is Zn^{2+} participation in the binding of both hGH and hPL to hPRLr with an enormously increased affinity of the ligand–receptor complex, supporting the recognition system of endocrine maintenance [90]. Additionally, the dimerization of hGH through Zn^{2+} elevates denaturation resistance during its storage in secretory granules hiding receptor binding epitopes. Upon hGH release from the pituitary gland, the availability of the hormone is strictly limited by intracellular labile Zn^{2+} [91]. Also, in the immunological process of lymphocyte development and antigen-dependent activation of mature T cells, one of the first-ever defined Zn^{2+}-mediated heterodimeric complexes, zinc clasp, brings lymphoid-specific, Src family protein-tyrosine kinase (Lck) kinase through cluster of differentiation 4 (CD4)/CD8α coreceptor binding to proximity to T cell receptor (TCR) [92,93]. Specificity and stability of heterocomplex formation are affected by structural and sequential CD4 molecular determinants [16,92]. Moreover, the balance between CD4 and CD4 homodimers was indicated to modulate the threshold of T cell activation [93]. The impact of Zn^{2+}, not only on immunity but also on brain physiology and pathology, is intensively studied, emphasizing the regulatory role of the ZPPI network.

The impact of Zn^{2+} was also demonstrated in protein polymerization, where its depletion causes vimentin filaments' reversible disassembly, influencing the cytoskeletal network, its architecture, signaling, and epithelial–mesenchymal transition [94]. Contrarily, Zn^{2+} inhibits polymerization of caspase recruitment domain-containing protein 9 (CARD9) and modulates interconversion between monomeric, domain-swapped dimeric, and filamentous states while restricting the capacity to form B cell lymphoma/leukemia 10 (Bcl10)-nucleating filaments [95]. The activated CARD9-Bcl10 signaling axis promotes downstream activation of immune factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and p38 mitogen-activated protein kinase (MAPK p38), as a response to a variety of pathogens. All the previously-mentioned examples seem to be just the tip of the iceberg in the intermingled network of the zinc interactome.

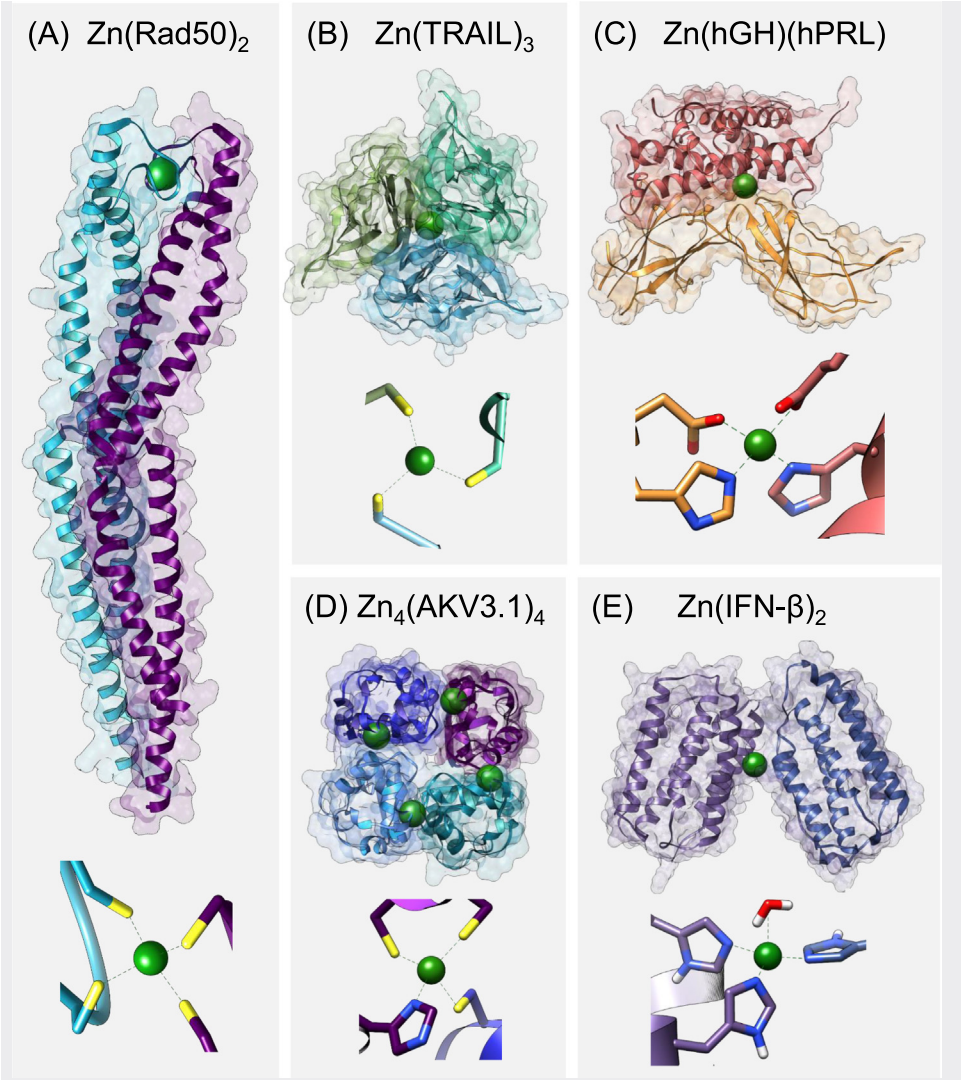

Figure I. Selected Multiprotein Complexes Containing Zinc Interfaces. Protein Data Bank (PDB) structure models present Zn^{2+}-mediated homodimers (A and E), heterodimer (C), homotrimer (B), and homotetramer (D). Insets below models show metal-binding amino acid residues. Zn^{2+} (green color) size is increased fivefold for clarity of presentation. Complex PDB ID: (A) 5gox, DNA repair protein RAD50; (B) 1dg6, APO2L/TNF-related apoptosis-inducing ligand (TRAIL); (C) 1bp3, prolactin receptor (hPRL) with growth hormone (hGH); (D) 3kvt, tetramerization domain from AKV3.1 (Shaw subfamily) voltage-gated potassium channel; (E) 1au1, interferon β.

structures. Based on the procedure applied here, 165 X-ray and NMR structures from the PDB database were derived and identified to contain potentially functional ZPPIs (Table S2 and Figures S1 and S2 in the supplemental information online), which is in agreement with a previous estimation [19]. Importantly, our goal was to select a representative database of ZPPIs, so the selected database should not be treated highly numerically. The way of selection and analysis is presented in more detail in the supplemental section S1 online. As a result, Figure 1 presents the timeline of deposition, organismal distribution, metal coordination mode, and functional assignment. The

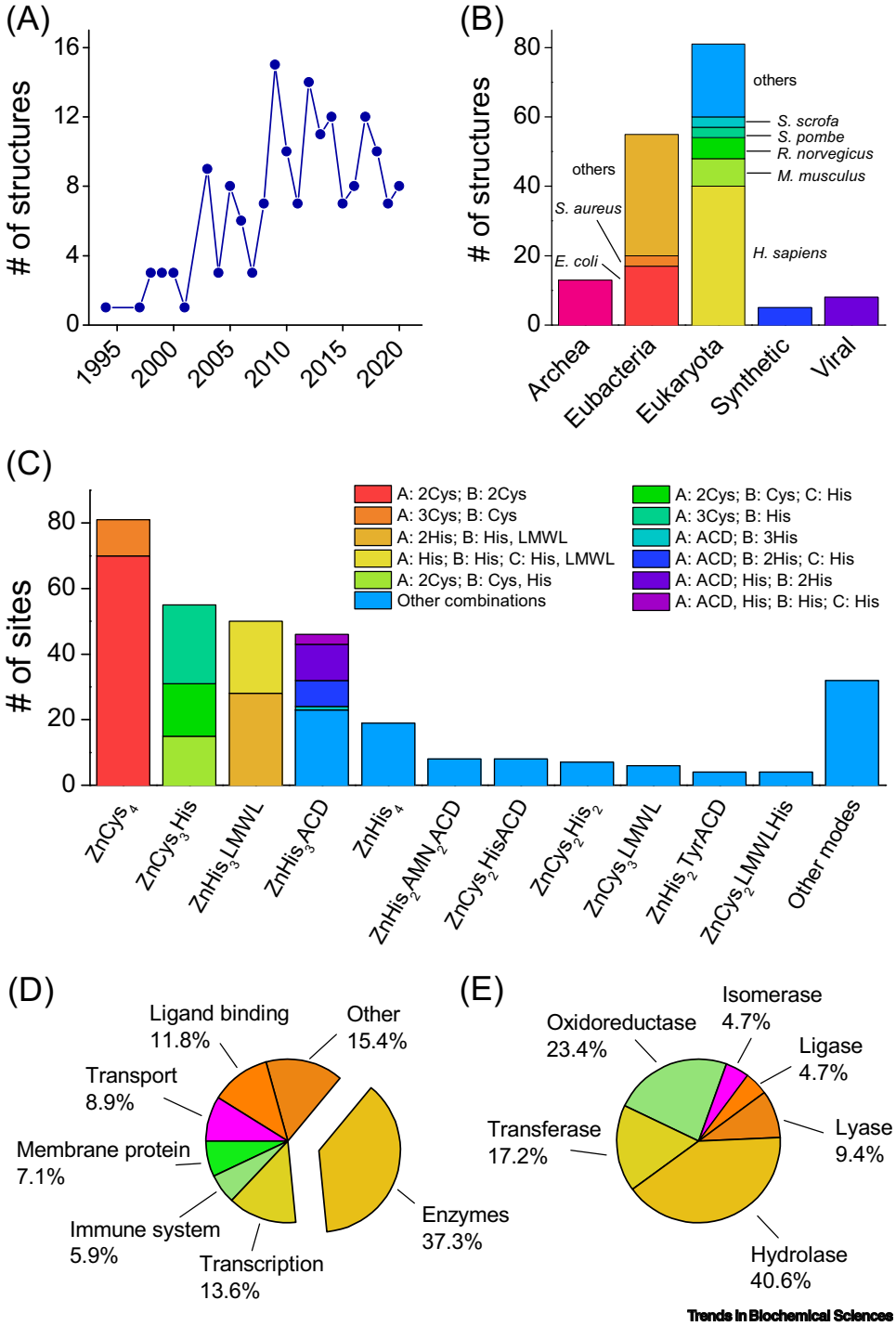

Figure 1. Descriptive Statistics of Protein Data Bank (PDB) Structures Containing Zn^{2+}-Involved Protein–Protein Interactions (ZPPIs) Found in Research Collaboratory for Structural Bioinformatics (RCSB). (A) The number of structures published per year. The graph shows a growing trend, despite large fluctuations caused by a small number of structures with ZPPI. (B) Structures' distribution is based on their origin. Species with the largest number of structures are shown. The number of structures does not reflect quantity in a particular group but rather research interest. *Homo sapiens* is

(Figure legend continued at the bottom of the next page.)

upward trend of deposited Zn^{2+}-containing protein interfaces over the last 20 years is noticeable, although the number of selected structures per year undergoes high fluctuations due to the low number of deposited structures (Figure 1A). The distribution of structures by gene source organism follows the general distribution of structures within RCSB PDB and corresponds to the interest and/or utility of a particular organism rather than the number of ZPPIs present in this organism (Figure 1B). The most common coordination number in the selected structures is four (~94%), followed by five (~4%), and six (~1%). In terms of the composition of the residues involved in the metal site, the most prevalent binding mode is Cys_4, followed by Cys_3His and His_3LMWL (Figure 1C). Previous analysis of all Zn^{2+}-binding sites did not distinguish the His_3LMWL group and, due to high variability of LMWL sites containing three histidyl residues, were likely to be classified as 'other' binding sites [28]. Nevertheless, the reason for the high occupancy of three histidyl nitrogens with one LMWL donor is hard to explain. A likely explanation is the size and spatial orientation of imidazole rings in those sites, which do not allow the fourth, bigger residue to bind, leaving room only for LMWL. Although high contents of Cys_4 and Cys_3His sites are observed for the ZincBind database and cysteinyl coordination is the most preferred one for structural binding sites in proteins, followed by histidinyl residues, the dominance of those two may be expected. Surprisingly, the most common coordination residues for identified ZPPIs turned out to be histidyl (37% of all ligands). In contrast to the cysteinyl one that is most common in all deposited zinc protein structures, in ZPPIs cysteinyl residues account for 26%, acidic residue content at zinc interfaces accounts for 19%, while water accounts for 10% of LMWL. The reason for the histidyl residue majority in ZPPIs is not clear. However, their large size allows main chains of interacting protein subunits to have a longer distance without or with minimal conformational change of protein molecules, which is demonstrated by the fast Zn^{2+} exchange and moderate affinity [16,18,29]. This is in contrast to cysteinyl residues, which are closer to each other at ZPPI interfaces and require a more significant change of protein structure upon metal binding, increasing the affinity of such a site (Box 3) [23]. Moreover, imidazole rings together with carboxylates of acidic residues allow for much more pronouncedly spatial binding orientation of interacting macromolecules, which is applied in supramolecular metalloprotein-based chemistry [29,30]. The impact of LMWLs is speculative and, besides some differences in filling the coordination sphere, they do not differ too much from structures of intraprotein Zn^{2+} complexes due to similar or the same crystallization conditions. LMWLs may differ between these two types of assemblies under physiological conditions regarding different functions, localization, and transient or permanent character. Various ligands may individually have an impact on Zn^{2+} geometry, affinity of metal-to-protein subunits, kinetics, and reactivity [11,17].

The functional assignment of PDB structures has been made according to the original reports (Figure 1D). The most common identified group is enzymes, where the high contribution of hydrolases and oxidoreductases is consistent with enzyme classes from the RCSB PDB database. This group can be further divided into two types, one where Zn^{2+} is directly involved in catalysis and the second where Zn^{2+} in an enzyme does not necessarily correspond to its catalytic properties (Figure S2 in the supplemental information online). We found examples of enzymes that utilize Zn^{2+} in ZPPI, such as L-histidinol dehydrogenase from *Medicago truncatula* [31] and 4-hydroxythreonine-4-phosphate dehydrogenase from *Paraburkholderia xenovorans* [32]. In the second group, Zn^{2+} is not involved in catalysis, but rather organizes the quaternary structure

the most representative organism, followed by *Escherichia coli*. (C) Metal coordination mode distribution. The most predominant coordination environment of Zn^{2+} in ZPPIs is 4Cys, followed by 3His, low molecular weight ligand (LMWL). The most predominant coordination number is four. Histidyl residue is the most predominant from all bound to Zn^{2+} in ZPPIs. (D) Distribution of PDB structures containing ZPPI is based on keywords assigned by authors of the structure (left). The most numerous group, which is the enzyme group, is divided into six classes of enzymes (right). Abbreviations: ACD, Glutamyl or aspartyl residue; AMN, glutaminyl or asparaginyl residue; *M. musculus*, *Mus musculus*; *R. norvegicus*; *Rattus norvegicus*; *S. aureus*, *Staphylococcus aureus*; *S. pombe*, *Schizosaccharomyces pombe*; *S. scrofa*, *Sus scrofa*.

Box 3. Stability of ZPPIs

Knowledge of the affinity of Zn^{2+} towards zinc proteins is critical for understanding their role in the zinc cellular maintenance. Low, high, or moderate affinity excludes Zn^{2+} as a potential cofactor, warrants protein saturation with Zn^{2+} under various cellular conditions, and suggests a transient function of Zn^{2+} in protein regulation, respectively. In consequence, protein saturation may either inhibit or activate protein function and the process relies on concentration of labile Zn^{2+} or its transients [16,96]. In typical Zn-P or standard PPI complexes, affinity differs in comparison to ZPPI, where both the two protein molecules and labile Zn^{2+} are limiting components (Table I). The application of the PPI model to metal-involved interaction with no consideration of Zn^{2+} concentration leads to serious errors, as in the case of Zn^{2+}-mediated CD4-Lck interaction. The initially reported K_d value (submicromolar) was overestimated, suggesting that the ZPPI is not physiologically relevant until proven to be much lower with the agreement of the identified picomolar range of labile Zn^{2+} concentration [92].

The stability of ZPPIs is driven by local and global factors: the first coordination sphere, conformational changes upon Zn^{2+} binding, and post-translational or chemical modifications that decrease the number of donors. It is also modulated when Zn^{2+} donors interact directly with other residues and the main chain or acid-base properties of active groups are affected by the local environment. Additionally, an interaction surface built by burying noncovalent interactions is the important selectivity factor of interacting partners in metal-mediated PPI [97]. Another important factor contributing to the structure and stability of the complex is **metal-coupled folding**. The formation of the secondary, tertiary, and even quaternary structures upon Zn^{2+} binding causes up to five to six orders of magnitude complex stability increase, as in the case of the zinc hook domain of Rad50 protein [61,62]. However, when Zn^{2+} assists in the organization, stabilization, or protection against proteases of ZPPI without folding events, Zn^{2+}-to-protein affinity remains relatively weak and depends on ligand types or other stabilizing factors. For example, Zn^{2+} binds to an insulin monomer with K_d of 0.4 μM [98], but it was found to bind to an insulin hexamer with a much higher picomolar affinity [99]. It was also shown that the binding of two Zn^{2+} ions to six insulin molecules from the hexamer is associated with the release of three protons, which is both enthalpically and entropically favored and elevates complex stability in the proton-coupled assembly mechanism.

Table I. Comparison of Complexation Equilibrium of Standard PPI and ZPPI [a,b]

	Standard PPI	ZPPI
Assembly reaction or Stabilizing structure reaction	P1 + P2 ⇆ P1P2 –	Zn^{2+} + P1 + P2 ⇆ ZnP1P2 or Zn^{2+} + P1P2 ⇆ ZnP1P2
Complex formation	$K_a = \frac{[P1–P2]}{[P1][P2]}$ [M^{-1}] –	$K_a^{12} = \frac{[ZnP1P2]}{[P1][P2][Zn^{2+}]}$ [M^{-2}] or $K_a^{*} = \frac{[ZnP1P2]}{[P1P2][Zn^{2+}]}$ [M^{-1}]
Complex dissociation	$K_d = \frac{[P1][P2]}{[P1P2]}$ [M]	$K_d^{12} = \frac{[P1][P2][Zn^{2+}]}{[ZnP1P2]}$ [M^{2}] or $K_d^{*} = \frac{[P1P2][Zn^{2+}]}{[ZnP1P2]}$ [M]

[a]A schematic reaction is presented where P1 and P2 stand for two protein units. K_a and K_d are the constants of protein–protein complex formation and dissociation, respectively, while K_a^{12} and K_d^{12} are the constants for ZPPI formation and dissociation, respectively (assembly reaction). Note that the ZPPI complex can be formed in a stepwise mechanism; first Zn^{2+} binds to P1 (K_a^1) and then formed ZnP1 binds to P2 (K_a^2)[b]. If Zn^{2+} binds to a preorganized P1P2 complex stabilizing the final ZPPI, then equilibria are described by K_a^* and K_d^* (stabilizing structure reaction).

[b]Constants describing stepwise ZPPI formation $K_a^1 = \frac{[ZnP1]}{[P1][Zn^{2+}]}$ [M^{-1}] and $K_a^2 = \frac{[ZnP1P2]}{[ZnP1][P2]}$ [M^{-1}].

and catalytic site like in the case of the 'Zn-link' in RNase E [33] or stabilizing Zn^{2+} in GET3-ATPase [34]. In enzymes Zn^{2+} may also have inhibiting properties; by asymmetric metalation Zn^{2+} is accountable for the half-of-site activity phenomenon in GloA2 from *Pseudomonas aeruginosa* [35]. Apart from the enzymatic ZPPIs, there are also pronounced examples of structural sites, for instance, Rad50 zinc hook (PDB ID: 5gox) or the assembly formed by the HIV-1 Tat complex (PDB ID: 5l1z). In the latter, a ZPPI is formed between human and viral proteins, indicating the importance of Zn^{2+} in host–pathogen interaction (Figure S1 in the supplemental information online). Recently reported Zn^{2+}-binding sites in the homodimeric ERp44, pH-sensitive chaperone cycling between endoplasmic reticulum (ER) and Golgi compartments and patrolling protein passing checkpoints turned out to control their oligomeric assembly. **Allosteric activation** of ERp44

revealed a regulatory role of Zn^{2+} for protein quality control in the early secretory pathway, enabling cycles of client binding, retrieval, and release [36]. The distribution of groups suggests that the existence of ZPPI does not correspond to a particular function of proteins, but rather indicates the versatility of Zn^{2+} to drive or modulate PPIs. The collected dataset is presented in Table S2 (see the supplemental information online). Currently, based solely on published structures, it is difficult to specify the exact role of Zn^{2+} in ZPPI, but with the help of additional functional and biochemical research, our understanding of the role Zn^{2+} plays in ZPPIs will surely increase.

Dynamic Zinc Network in Intersystemic Communication

Cellular Zn^{2+}, to play an enormous number of functions, must be strictly controlled by the homeostatic machinery, which in general includes its transport, storage, and buffering to keep intracellular labile Zn^{2+} in a proper concentration ($[Zn^{2+}]_i$), which varies depending on the cell type and determination method from 10^{-11} to 10^{-9} M (see Figure I in Box 1 and Table S1 in the supplemental information online) [17,21]. Zn^{2+} influx and efflux, zinc transients, may affect the regulatory machinery locally and timely with significant consequences when transporters' overexpression is not sufficiently spatiotemporally matched or when buffering capacity is exceeded [13,37]. This influences ZPPIs, which occur transiently and demonstrate a moderate affinity for Zn^{2+}. It is therefore important to underline that the Zn^{2+} influx shifts the equilibrium towards ZPPI formation and the efflux to non-interacting complex components. In a transient initiation, a trigger may be a different molecule or Zn^{2+} itself. Primary sources of intracellular Zn^{2+} release are cellular compartments that demonstrate the involvement of Zn^{2+} in the biosynthesis, export, and elimination of cellularly important molecules [13]. Stimulation of receptors by their ligands, growth factors, and cytokines leads to a change in their concentration, thus influencing the number of signaling pathways (Table 1) [38]. The most prominent example of a zinc transient is a zinc wave, a gated release of Zn^{2+} from the ER and Golgi (where $[Zn^{2+}]_i$, depending on the report, varies from 10^{-13} to 10^{-9} M), mediated by ZIP7 after mast cell or lymphocyte stimulation (see Figure I in Box 1 and Table S1 in the supplemental information online) [39]. It causes a rapid increase of $[Zn^{2+}]_i$ and modulates the ERK/MAPK pathway, supporting the role of Zn^{2+} as a secondary messenger. However, the lack of mechanistic details of its action for most of the identified zinc transients depicts another gray area of ZPPIs

Table 1. Intracellular Zn^{2+} ($[Zn^{2+}]_i$) Transients (Short- and Long-Term) among Different Cell Types

Stimulation	Receptor	Cell type	Source of $[Zn^{2+}]_i$ increase	Mechanism/effect	Refs
LPS	TLR	Monocytes	Intracellular stores	p38 MAPK, ERK1/2, NF-κB, abrogated release of TNFα	[100]
IgE	FcεRI	Mast cells	ER (ZIP7) translocation of PKC and NF-κB	Mast cell activation, allergic reactions, degranulation, cytokine production	[101]
Receptor crosslinking	FcεRI	Mast cells, lymphocytes	ER ('zinc wave')	ERK/IP3, MAPK/ERK	[39]
CD40	BCR	Lymphoblastoid cell lines	ZIP7 increase	ZIP7 phosphorylation	[102]
Anti-IgM antibody		B cells	Golgi (ZIP9)	Akt/ERK phosphorylation, BCR signaling	[103]
IL-2	IL-2R	T cells	Lysosomes	Erk1/2 phosphorylation, proliferation	[104]
Zn^{2+}	EGFR	Breast cancer cells	ZIP7 EGFR activation	EGFR/IGF-I/Src	[105]
Zn^{2+}	ZIP6 (channel)		Zinc influx	GSK-3β phosphorylation, STAT3 activation, adherence genes regulation	[106]
Zn^{2+}, EGF, ionomycin	n.i., EGFR		ER (ZIP7 phosphorylation)	Akt/ERK phosphorylation	[107]
IP3, thapsigargin	IP_3R (ER)	Cortical neurons	ER	n.i.	[108]

Abbreviations: Akt, Protein kinase B; BCR, B cell receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FcεRI, Fcε receptor I; GSK, glycogen synthase kinase; Ig, immunoglobulin; IGF, insulin-like growth factor; IL, interleukin; IP3, inositol 1,4,5-triphosphate; IP_3R, inositol triphosphate receptor; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; n.i., not identified; PKC, protein kinase C; TLR, Toll-like receptor; TNF, tumor necrosis factor;; STAT, signal transducer and activator of transcription.

that needs to be explored further. To date, an interplay between inhibition of phosphatases and the activity of kinases is suggested as a major zinc mode of action [40].

The relation of intra- and extracellular Zn^{2+} is indispensable to decipher the molecular role of Zn^{2+} in protein–protein networking where the target is a zinc-sensing receptor, ZnR (GPR39), with an unknown mechanism of action [41]. However, it has been found that extracellular changes in Zn^{2+} concentrations mostly result from Zn^{2+} release from vesicles found in high concentrations (e.g., in the brain, mammary and salivary glands, and digestive system) [42–44] (see Figure I in Box 1). Zn^{2+} released, for example, upon synapse stimulation, modulates all major neuronal membrane transporters [dopamine, *N*-methyl-D-aspartate (NMDA), glycine, γ-aminobutyric acid (GABA)] [13,45–47], where thorough mechanistic analysis of GluN1/GluN2A NMDA revealed recently how the zinc site, although intraprotein, modulates a large ensemble of complex conformations [48]. The regulatory role of Zn^{2+} in synaptic transmission and plasticity has been shown to affect long-term potentiation (mechanism of learning and memory) as well as modulation of hypothalamic–pituitary–adrenal axis activity responsible for cognitive and emotional function [49,50]. Although most of the molecular targets seem to be still not identified, the family of Shank proteins from the actin-based cytoskeleton is stabilized interfacially by Zn^{2+} [51]. Large sheets composed of helical fibers stacked side by side form a platform for the construction of the postsynaptic density complex where Zn^{2+} regulates cytomatrix plasticity [52].

Apart from the prominent Zn^{2+} involvement in the nervous system, its influence has also been demonstrated for endocrine, immune, cardiovascular, digestive, reproductive, and skeletal systems. Moreover, with an increasing level of organization, from organelles in a cell to the organism composed of different organs, the intermingled networks of the zinc interactome appear to be more evident. They mutually affect each other and their functioning is inevitably impaired when isolated. An example of intersystemic communication with Zn^{2+} is the pancreas and liver. Hexameric insulin molecules capture Zn^{2+} in granules of pancreatic β-cells, where total Zn^{2+} concentration reaches 10–20 mM (Table S1 in the supplemental information online) [44]. Under insulin secretion, released Zn^{2+} inhibits glucagon release from alpha-cells. At the same time, the endocrine signal affects blood insulin clearance by the liver through inhibition of insulin internalization. ZnT8 has been established as a pivotal contributor to the insulin level of delivery to the liver and optimizer of its effect on glucose metabolism [53]. Another illustration of intersystemic Zn^{2+} fluctuation is the zinc spark: the captured image of billions of Zn^{2+} ions released from the mammalian egg upon fertilization [54,55]. Ejection of Zn^{2+} into the extracellular milieu in a series of coordinated events mediates a decrease in intracellular Zn^{2+} content, which is crucial to the transition of a meiotic cell into a mitotic one and progression of the cell cycle.

Zn^{2+} fluctuations also contribute to thyroid and mammary gland maintenance, influencing the architecture of glands, secretory capacity, and lactation performance [56]. When deregulated they result in increased prolactin receptor trafficking, which as a ZPPI with lactogenic hormones, such as human growth hormone (hGH), lead to substantial changes of receptor–ligand complex formation. Additionally, hGH and insulin-like growth factor-I (IGF-I) endocrine factors from the pituitary gland and liver are combined in the GH-IGF axis that regulates somatic growth. Under zinc deficiency, reduced GH (also IGF-1 levels) cannot be reversed and the growth retardation phenotype persists [57]. Cardiovascular system regulation with Zn^{2+}-involved pathways is strongly grounded in sulfur-based chemistry, where it is released from proteins depending on the oxidative condition of a cell. The involvement of Zn^{2+} release in excitation–contraction and excitation–transcription cycling of mammalian cardiomyocytes has just started to be investigated, in contrast to well-established Ca^{2+} changes. But lately, the reported rise in **labile Zn^{2+} concentration**, being more than ten times higher than the Ca^{2+} rise, together with the main receptor of Ca^{2+} transport being highly activated by Zn^{2+}, greatly supports the role of Zn^{2+} in shaping Ca^{2+} dynamics [58].

To sum up, fluctuations of Zn^{2+} play a major role in the physiological functions of virtually all organ systems; however, molecular and mechanistic aspects of such regulation are still poorly understood. This unexplored territory possesses many challenges and creates a new area of research. Attempts such as the first global transcriptional screening of genes involved in zinc-dependent processes and functions surely will shed more light on the matter [59].

Factors Driving Zn^{2+}-Mediated PPIs

Taking into account the structural diversity in ZPPIs and not fully explored Zn^{2+}-dependent cellular network, one may say that metal-to-protein components' affinity together with rates of formation and dissociation might be critical factors that separate their modulatory role from structural functions (Figure 2A–C) [13,15,16]. The mechanisms of Zn^{2+} interaction with protein components, forming together the ZPPI complex, can be therefore categorized into metal-driven and metal-stabilized, where the affinity and the surface area of PPI are interwoven (Figure 2D,E). It is worth noting that redox state, local $[Zn^{2+}]_i$, and protein component concentrations additionally interplay with metal affinity and, consequently, the Zn^{2+}-mediated complex assembly process. Post-translational modification of certain amino acid residues in a protein subunit or in a Zn^{2+}-binding residue may influence ZPPI formation or transient/permanent function by analogy to intraprotein Zn^{2+} complexes due to conformational change of the protein subunit or by affecting metal-to-protein affinity (Figure 2F). Currently the literature lacks accounts of such relations besides disulfide bond formation/break in circadian proteins [13,16,17,21,60].

Most of the identified and structurally characterized ZPPIs contain an extensive noncovalent electrostatic and hydrophobic network occurring between protein molecules that outcompete the energetic cost attributed to the loss of entropy and dehydration of protein surfaces [61,62]. They are formed in close proximity and far from the Zn^{2+}-defining surface of interprotein interaction. The binding of a metal ion at the protein–protein interface much more strongly contributes to the complex formation thermodynamics. Bonds formed between Zn^{2+} and amino acid side groups have either covalent or semi-covalent character. The proportion of these two types of interaction in ZPPIs seems to have an important impact on the complex assembly mechanism and, potentially, its function. In ZPPIs, where electrostatic and hydrophobic interactions bury a large surface area, 1500–10 000 $Å^2$ typically for most PPIs, mechanistically protein–protein association may occur without Zn^{2+} participation [11,17,19]. Metal coordination to the preorganized site increases the total stability of the whole complex, resistance to certain chemicals and proteases of the whole complex, or very subtly adjusts the orientation of the molecules, as in the case of nitric oxide synthases isoenzymes (NOSs). Endothelial NOS (eNOS) active dimeric form is stabilized by allosteric calmodulin binding to the reductase domain, cofactor binding pocket, and strategically located zinc site maintaining the integrity [63]. However, it has been shown that depletion of Zn^{2+} from active neuronal NOS (nNOS) homodimer does not result in inactive protein; however, metal dissociation is the most plausible explanation of decreased complex stability and components' dissociation in time [64]. When the noncovalent surface interaction area decreases, the force of impact comes from metal–ligand coordination bonds and associated conformational changes. A good example is the thermodynamically stable zinc hook domain, with a very small interaction area formed at the apex of the coiled coils of two Rad50 molecules from *Pyrococcus furiosus* being part of the MRN(X) complex [i.e., the Mre11, Rad50, Nbs1 (Xrs2) complex] responsible for DNA double-strand break recognition and repair [18,65]. Zn^{2+} binding to two Cys residues from each protomer causes their local folding, which is a driving force for its extreme stability (Box 3) [61,62,66]. Interestingly, Zn^{2+} affinity to NOSs is comparable to Rad50, yet there are major differences in the interaction area. This indicates that, despite the difference in ZPPI complex assembly mechanisms, similar affinity can be reached due to various factors, extensive PPI based on numerous noncovalent interactions, or significant structural changes induced by Zn^{2+}.

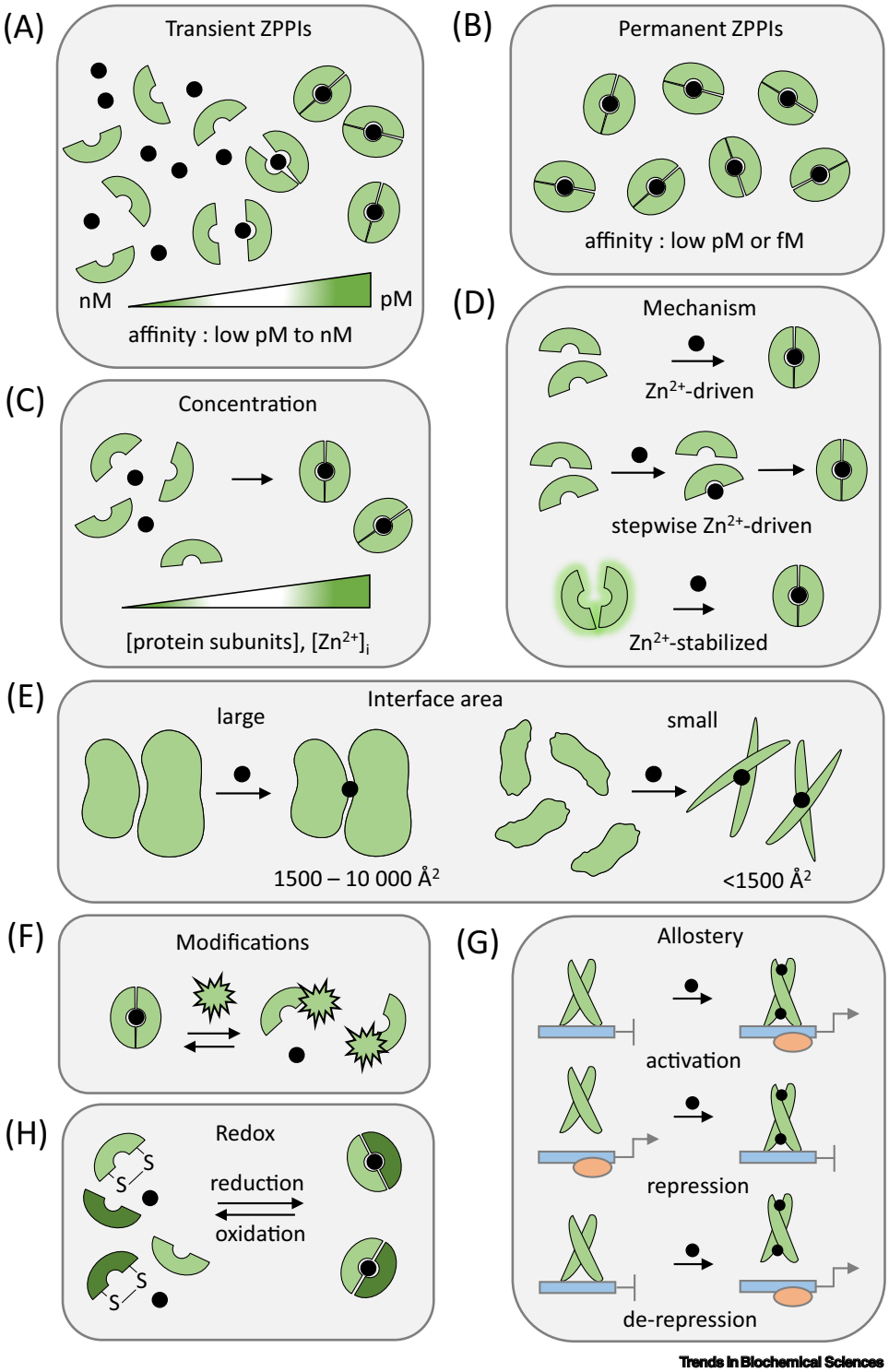

Figure 2. Schematic Representation of the Mechanisms and Factors Driving Zn^{2+}-Involved Protein–Protein Interactions (ZPPIs). (A) Formation/dissociation of transient assemblies depends on labile Zn^{2+} concentration and components' affinities to Zn^{2+} that are in pico- to nanomolar range (see Table I in Box 3). (B) Permanent ZPPIs are formed

(Figure legend continued at the bottom of the next page.)

Regulatory sites present in ZPPIs work somewhere between the earlier-discussed examples. Zn^{2+} binds to interacting protein molecules with moderate affinity, which is reached by limited interactions buried in the interface or limited structural changes associated with metal binding. It is worth noting that Zn^{2+} affinity (K_d) for its site in regulatory ZPPIs must be comparable to labile Zn^{2+} concentrations (transients) that either modulate protein assembly or cause subtle (intimate allosteric) changes in complex conformation that can play a specific function. The indirect influence of a metal ion in an allosteric mechanism is observable in dimeric transcriptional metalloregulators in which the metal ion either activates or inhibits DNA binding to activate, repress, or de-repress transcription (Figure 2G). Monomers in these homodimers interact via noncovalent interactions in such a way as to preorganize two metal binding sites. Metal sites with distinct coordination chemistries and metal specificities evolved in different places to achieve new adaptive responses [67]. Although so far mostly investigated from bacterial sources, such as CzrA and ZitR, metalloregulatory proteins show a thermodynamically complicated network of perturbations and changes of residue motions that led to interconversion and subsequent change in their functionality [68,69]. Additionally, the cooperativity of two Zn^{2+}-binding sites, as in histidine switch-mediated cooperation in ZitR, adjusts DNA-binding affinity in response to a broad range of zinc fluctuation [68]. Zn^{2+}-mediated and allosterically driven PPI may occur without an interprotein metal ion. Its binding to one protomer may induce the proper tertiary fold that allows specific interaction with its partner, as has been shown in the case of the DENR-MTC-1 complex. The role of Zn^{2+} in ribosomal complex formation gives an insight into the mechanisms of noncanonical translation initiation, re-initiation, and regulation of ribosomal recycling [70]. An allosteric influence can also be achieved by post-translational modifications for both PPI and ZPPI, where phosphorylation is the most investigated covalent modification so far [71]. For the membrane-proximal PPIs, where lipid-binding events take place, allosteric modulation of integral membrane proteins and soluble protein of the PPI has also been reported in recent years [72].

Zn^{2+}-mediated interaction may occur in a redox-dependent manner, as in the case of the circadian clock proteins (Figure 2H). It has been shown that the reduction of the disulfide bridge in mCRY1, located near a metal docking site, enhances the flexibility of the C-terminal lid and promotes PER2 binding. Zn^{2+} coordinates to the histidyl and one of the reduced cysteinyl residues of CRY1, and the remaining two positions are filled by two cysteinyl resides of PER2, forming a Zn^{2+}-stabilized heterodimer [60]. The CRY1/PER2 complex, therefore, acts as a sensor of the rhythmically changing redox state of the mammalian cell, which was proposed to be involved in driving circadian rhythms. Moreover, it has been shown that Zn^{2+} binds to its interprotein binding site with low nanomolar affinity (measured by isothermal titration calorimetry), which corresponds to the determined labile Zn^{2+} concentration range. This strongly suggests that the circadian clock can be modulated by both factors, redox and available Zn^{2+} changes. Still, it is not clear what is oxidized, the Zn^{2+}-thiolate core in the CRY1/PER2 complex or CRY1 itself after Zn^{2+} dissociation. Regardless, this example shows that affinity-driven ZPPI formation can be enhanced or disrupted by other cellularly occurring reactions [73].

Due to a number of factors, some of which have been mentioned in this article, cells gained the ability to maintain the intrinsically dynamic network of PPIs. Not only Zn^{2+}-to-protein affinity and

even at low picomolar and femtomolar labile Zn^{2+} concentration. (C) Impact of subtle labile Zn^{2+} and protein subunit concentration on ZPPI formation/dissociation. (D) Two major assembly mechanisms present in ZPPIs. (E) Different interface areas in ZPPIs influencing complex stability. (F) Influence of post-translational or chemical modifications on ZPPI formation/dissociation. (G) Allosteric changes upon Zn^{2+} binding to metalloregulators modulate complex functionality. Blue rod and orange oval indicate DNA chain and polymerase, respectively. (H) Redox reaction controls protomer assembly. The reduction of protein subunits results in the formation of functional ZPPI, while oxidation causes protomers and Zn^{2+} dissociation.

availability of Zn^{2+} itself but also the local abundance of proteins from the zinc interactome network affects the formation of functional Zn^{2+} complexes due to the law of mass action. It has been therefore demonstrated that the absolute molar fraction of a ZnL-type complex is independent of protein concentration and depends on both metal affinity and labile Zn^{2+}. In contrast, the formation of ZnL_n ($n \geq 2$) complexes typically for ZPPIs occurs additionally in a protein-concentration manner [16]. In other words, at a certain $[Zn^{2+}]_i$, the fraction of a formed ZPPI increases or decreases within subunit concentration changes. Secondly, even small or local changes in $[Zn^{2+}]_i$ may significantly change the fraction of functional Zn^{2+} complex, depending on its architecture, function, and subcellular localization. Additional factors mentioned earlier, such as post-translational modification, redox potential, or even pH (different for various compartments), and the impact of reactive species or LMWL, may interplay with all the earlier-mentioned factors, including metal affinity. This wide dependence network is especially important for ZPPI complexes formed on the membrane or solid particle surfaces where the local concentration may differ from that of the cytosol [74]. Moreover, even in the cytosol, local labile Zn^{2+} transients may differently affect the formation of intra- and intermolecular Zn^{2+} complexes. Overall, Zn^{2+}-mediated interactions and their cellular dynamics are driven by numerous factors, from surface specificity, through structural/conformational changes, to specifically occurring and modulating reactions.

Concluding Remarks

Understanding the role of ZPPIs in a whole zinc interactome requires not only their architectural investigations but also thermodynamics, kinetics, and reactivity/functionality studies in a dynamically changing pool of labile Zn^{2+}, which simply differentiates permanent from transient Zn^{2+}-mediated interactions. Zn^{2+}-involved protein interactions have been recognized as a source of protein structural and enzymatic modulators. However, their role at protein–protein complex interfaces is not yet well described, mostly due to recognition obstacles regarding their natural transient character and thus loss of Zn^{2+} outside the natural environment, mostly in the process of protein preparation and purification (see Outstanding Questions). It is also important to note that interfacial Zn^{2+} found in numerous protein crystal structures is frequently artificial due to protein production in the host organism or cocrystallization in media containing Zn^{2+}. Therefore, our current knowledge regarding the existence of functional interprotein zinc sites is based on cellular and biochemical studies, which are consistently structurally supported. Although the structural protein database might be a source of artifacts, it is still very helpful for finding new functional ZPPIs. Screened records presented here are either known or not yet functionally recognized ZPPIs, which we hope will inspire future investigation. To understand the function of interprotein sites and their formation/dissociation processes, an additional toolset of high throughput techniques should be used, including, for instance, global gene expression, proteome and labile Zn^{2+} analysis supported by mass spectrometry and sensitive fluorescent methods/sensors in different cellular conditions (e.g., zinc excess and its deficiency, or physiological and pathological stages) (see Outstanding Questions). Only this way do we have a chance to capture not only thermodynamically stable complexes but also transient interactions occurring under specific conditions. The intersystemic Zn^{2+}-dependent network presented here illustrates how far we have come in understanding the role of Zn^{2+}-mediated protein–protein communication, signaling, and complex assembly in a global zinc interactome.

Outstanding Questions

What are the functions of zinc sites at protein–protein interfaces? How do these functions correspond to intraprotein zinc sites?

How many ZPPIs are present in the human zinc interactome? Is it possible to predict them using any combined experimental-bioinformatic tool?

What is the specificity of Zn^{2+} coordination in ZPPIs? Are particular amino acid residues prone to form functionally distinct ZPPIs?

What structural factors affect ZPPIs formation and control their thermodynamics and kinetics?

How can we confirm the existence of ZPPIs?

Taking into account available data with combination of gene expression analysis and zinc status, can we estimate changes in ZPPI profiles in cells under zinc supplementation, deficiency, or pathogenic conditions?

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