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Review

Intrinsic disorder and fuzzy interactions drive multiple functions of HMGB1

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HMGB1, a multitasking protein, is scrutinized here through the lens of the 'fuzzy interactions' driven by its intrinsically disordered regions (IDRs). Although the multiple intracellular and extracellular functions of this protein have been studied for decades, viewing HMGB1 as fuzzy and dynamic provides a novel perspective. Recent breakthroughs emphasize the crucial role of its IDRs, especially the acidic C-terminal tail, in mediating dynamic multivalent interactions. This fuzziness enables HMGB1 to modulate DNA and chromatin binding, to chaperone other proteins such as p53, and to tune inflammatory signals via receptors such as TLR4 and CXCR4. Understanding the fuzzy nature of HMGB1 unlocks new therapeutic strategies targeting both its structured and unstructured regions to tackle a range of diseases.

Highlights

Fuzzy interactions mediated by intrinsically disordered regions (IDRs) allow dynamic regulation and interaction diversity across various cellular processes relevant to biological activity.

High mobility group box 1 (HMGB1) protein, a multitasking molecule involved in diverse intracellular and extracellular processes and several human diseases, is intrinsically fuzzy and forms fuzzy complexes.

We delve into how the conformational malleability of HMGB1 provided by its IDRs underpins its ability to engage with multiple partners and perform diverse functions across different cellular compartments and conditions.

Fuzziness appears to be essential in modulating HMGB1 diverse functions. Understanding HMGB1 functions through the lens of fuzziness provides a novel perspective.

Mastering multifunctionality through intrinsic disorder

High mobility group box 1 (**HMGB1**; see Glossary) stands out as prototypical example of a protein with multiple functions and multiple interactors. HMGB1 is an 'eclectic' protein whose post-translational modifications, oxidation states, and variety of functions in different cellular compartments have been the subject of extensive studies and reviews in the past 50 years [1–4]. Perhaps not surprisingly, we and others have gathered evidence that HMGB1 engages in **fuzzy interactions**, and HMGB1 is included in the database of fuzzy proteins [5]. Notably, the fuzziness of HMGB1 is associated with both its intracellular function as a chromatin-binding protein and its extracellular functions as a signaling molecule.

The term fuzzy stems from the fuzzy logic of variables that can assume values between true (= 1) and false (= 0) rather than complying with the strict Boolean logic of either true or false [6]. Protein fuzziness is intertwined with structural order that defines a spectrum from entirely folded to unfolded [7,8]. Polypeptide chains in protein domains are folded in one or a few stable conformations, whereas **intrinsically disordered regions (IDRs)** adopt dynamic, interconverting conformations that lack a stable 3D structure. The estimated total size of IDRs is >30% of the total length (in amino acids) of the eukaryotic proteome [9,10], which suggests that they are extensively used to perform physiological functions in eukaryotes. Given their crucial roles in cellular processes and their involvement in diseases such as cancer and neurodegeneration, and because recent technological advances have enabled deeper insights into their structure and function, IDRs are increasingly recognized as key players in both basic biology and therapeutic development.

IDRs are generally characterized by low-complexity sequences, often containing charged residues [11–14], and they often play a major role in the recognition of interacting molecules by their host proteins [15]. Upon interaction, IDRs can cover a continuous spectrum of structural states, ranging from retaining full disorder to adopting a stable fold. In the former case, they

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can exchange between multiple conformations, giving rise to so-called **fuzzy complexes** where the structural multiplicity of the distinct bound states is relevant to the biological activity of the entire complex [7,16–18]. Of importance, the conformational malleability of IDRs allows them to engage with multiple partners and in different cellular contexts, thus broadening the interaction diversity of their host protein [19]. The aim of this review is to spotlight the fuzzy interactions in which HMGB1 is known to be involved in and to showcase HMGB1 as an example of a protein whose multifaceted activities are propelled by fuzziness.

HMGB1 is a multitasking fuzzy protein

The functional versatility of HMGB1

HMGB1 is a non-histone nuclear protein that is widely expressed in mammalian cells and tissues and exerts multiple functions according to its subcellular location [1,2]. HMGB1 is very abundant in the nucleus, where it acts as a DNA chaperone by binding to distorted DNA structures [20], including Holliday junctions, DNA duplexes containing an indel on one strand, and G-quadruplexes, and generates transient distortions on linear DNA that can for example facilitate nucleosome formation [1,2,21]. It can also shuttle from the nucleus to the cytoplasm where it promotes **autophagy** by binding to beclin-1, thus displacing beclin-2 [22]. Finally, in stress conditions, it can be actively or passively released into the extracellular space where it acts as a **damage-associated molecular pattern (DAMP)** by signaling cell death or distress to the immune system [23].

HMGB1 is also a redox-sensitive protein: it contains three cysteines that can be either fully reduced (frHMGB1) or partially oxidized through a Cys23–Cys45 intramolecular disulfide bond in which Cys106 maintains the thiol form (dsHMGB1) or is fully oxidized by sulfonation [2,24] (Figure 1A). frHMGB1 is typically released during early inflammation, tissue repair, and sterile inflammation, and exerts chemoattractive effects by activating CXCR4, a G protein-coupled receptor (GPCR) involved in immune responses, via CXCL12, a chemokine which binds to CXCR4 [25]. dsHMGB1, which is more commonly associated with late and chronic inflammatory responses [1,2], binds to Toll-like receptor 4 in complex with myeloid differentiation factor 2 (TLR4•MD2), which is also crucial for the innate immune system to respond to bacterial infections [26]. In these two interactions, frHMGB1 and dsHMGB1 are mutually exclusive, and fully oxidized HMGB1 is inert [25]. The receptor for advanced glycation end-products (RAGE) may bind to all redox forms of HMGB1, and HMGB1 binding to RAGE prompts autophagy [1,27].

The structural fuzziness of HMGB1

The functional versatility of HMGB1 depends not only on its subcellular localization and oxidation state but also on its structural and dynamic features. HMGB1 is a protein of 214 amino acids (aa) and contains both structured and IDR regions. In detail, it contains two structurally conserved HMG boxes (box A, residues 14–77; box B, residues 101–163) composed of three α-helices (1–3) arranged in an L shape, and three IDRs covering 40% of the whole protein (Figure 1A,B). One IDR corresponds to the linker connecting the HMG box domains (24 aa); another IDR, immediately after box B, contains 21 aa rich in lysines and is thus commonly defined as the basic linker, and an acidic C-terminal tail (30 aa) (acidic IDR) that is exclusively composed of aspartate (D) and glutamate (E) residues. Of note, the Alphafold Protein Database (AF-P09429-F1-v4) predicts the last 50 residues of HMGB1 with a very low model confidence score (predicted local distance difference test, pLDDT = <50), consistent with the intrinsically disordered nature of the acidic IDR. Also important is the phylogenetic conservation of the acidic IDR in HMGB1 molecules of different species ranging from human to invertebrates, suggesting that its presence is not ancillary but plays an important role in HMGB1 functions [28]. Finally, although the N-terminus (14 aa) lacks

Glossary

Autoinhibition: the intramolecular inhibition of a protein activity by one of its own domains or regions. The acidic IDR of HMGB1 causes autoinhibition by transiently interacting with the basic DNA-binding surfaces of the HMG boxes and the linker IDR, affecting binding with its partners.
Autophagy: a conserved lysosomal degradation process that recycles cytoplasmic components, including damaged organelles and proteins, to maintain cellular homeostasis and adapt to metabolic or stress conditions.
Chaperone-like activity: the ability of a protein to assist the proper folding or assembly of other molecules. HMGB1 exhibits chaperone-like activity, for example in facilitating the binding of p53 to its cognate DNA target site.
Conformational heterogeneity: in intrinsically disordered proteins (IDPs), conformational heterogeneity refers to the presence of a diverse range of rapidly interchanging conformations or microstates. This results in a dynamic conformational ensemble where the protein does not adopt a single fixed structure but instead exists in multiple interconverting flexible forms.
Damage-associated molecular patterns (DAMPs): endogenous molecules released by stressed, injured, or dying cells that signal danger to the immune system. They are recognized by pattern recognition receptors such as Toll-like receptors (TLRs), NOD-like receptors, and receptor for advanced glycation end-products (RAGE) that activate immune cells and drive inflammation, contributing to both defense and disease.
Fuzzy complexes: biological complexes where one or both partners present structural ambiguity or multiplicity. In fuzzy complexes, proteins can adopt a wide range of structural order depending on the cellular context. Structural multiplicity of the bound states is relevant to the biological activity of the entire complex.
Fuzzy interactions: dynamic and conformationally heterogeneous contacts within or between biomolecules, often mediated by IDRs. These interactions allow structural multiplicity relevant to the biological activity.

a defined secondary structure and is predicted to be disordered, it exhibits reduced dynamics because of transient interactions with helix α3 of box A [29].

Several solution-state biophysical studies, including solution-state nuclear magnetic resonance (NMR) methods, small-angle X-ray scattering (SAXS), circular dichroism (CD), isothermal titration calorimetry (ITC), and molecular dynamics (MD) simulations are all in agreement in showing that frHMGB1 is **conformationally heterogeneous** and populates an ensemble of different microstates [30–33]. On the one hand, the IDR bridging the two HMG boxes allows the two domains to behave independently, with essentially the only constraint of being tethered to each other; on the other hand, the C-terminal acidic IDR transiently associates with the rest of the protein without adopting a precise conformation. As a result, macroscopically, HMGB1 oscillates between a collapsed and an open form (Figure 1C) in which the acidic IDR is sandwiched between the two HMG boxes via weak **multivalent** intramolecular **long-range electrostatic interactions** between the D/E repeats of the acidic C-terminal IDR, the basic HMG boxes, and the interdomain connecting IDR. Removal of the acidic IDR shifts the equilibrium towards an open and less stable form. Overall, the high number of conformational microscopic states within HMGB1, as is often observed in the molecular recognition of intrinsically disordered proteins (IDPs) [34], provides an entropic benefit that minimizes the conformational entropy loss upon the acidic IDR binding and increases the modes of interaction with different molecules [33]. In particular, the acidic IDR plays a significant role in modulating protein–protein interactions by providing flexibility and adaptability. This enables HMGB1 to transiently interact with a variety of binding partners across different cellular environments, making it a versatile mediator of multiple signaling pathways and cellular responses.

As a prototypical fuzzy molecule (Figure 1B) [8,35], HMGB1 participates in numerous intermolecular interactions where its intrinsic disorder is preserved when binding to various partners such as proteins [36], nucleic acids [33], or small molecules [37] that directly influence its biological functions. In the following sections we explore the complex interactions involving HMGB1, focusing on how the transient interactions of its acidic IDR can either enhance or inhibit binding in different cellular contexts. We primarily concentrate on HMGB1 interactions with chromatin and extracellular molecules that are best characterized at the molecular level.

Fuzzy interactions between HMGB1 and chromatin are driven by the acidic IDR

The autoinhibitory role of the acidic IDR in frHMGB1–DNA interactions

IDPs and IDRs are particularly abundant in chromatin and chromatin-associated proteins, where they often drive crucial intermolecular assemblies involving protein–protein and protein–nucleic acid interactions and the formation of phase-separated condensates [38–40]. IDRs/IDPs are frequently characterized by a high content of charged amino acids that may favor both interactions with the solvent and conformational multiplicity [11–13]. D/E repeats are highly represented in DNA/RNA-binding proteins within the mammalian proteome [14], where they can have **chaperone-like activity** [41,42] or cause protein **autoinhibition** via transient intramolecular electrostatic interactions with positively charged domains, thus competing for binding to nucleic acids [43,44].

As an architectural chromatin-bound protein, frHMGB1 facilitates the formation of protein–DNA assemblies involved in transcription, recombination, DNA repair, and chromatin remodeling [45]. Its ability to bend DNA in a non-sequence-specific manner is central to its nuclear function, and the acidic IDR modulates this ability. A study in which single-molecule fluorescence resonance energy transfer (FRET) was coupled to frHMGB1 truncations and mutants showed that the full-length protein bends DNA more than the individual HMG boxes, but less than the protein without the acidic IDR. The authors proposed a model in which the acidic IDR serves as an intramolecular damper that modulates the interaction of the B box with DNA [43].

High mobility group box 1 (HMGB1): a non-histone nuclear protein with multiple intracellular and extracellular functions including DNA binding, autophagy, and inflammation. It is a prototypical example of a protein with multiple functions and multiple interactors. Its functional versatility depends on its subcellular localization, oxidation state, and structural/dynamic features.

Intrinsically disordered regions (IDRs): regions within a protein that adopt dynamic interconverting conformations that lack a stable 3D structure. IDRs are often characterized by low-complexity sequences containing charged residues and play a major role in the recognition of interacting molecules.

Liquid–liquid phase separation (LLPS): a process by which biomolecules condense into dynamic membraneless organelles. The polyelectrolyte characteristics of the acidic IDR of HMGB1 confer the ability to undergo LLPS.

Long-range electrostatic interactions: electrostatic forces between charged amino acids that extend over considerable distances. These interactions play a crucial role in determining the structural and dynamic properties of IDRs, and influence their conformational diversity and functional behavior. The acidic IDR of HMGB1 acts as a binding cloud that recruits the positively charged CXCL12 via long-range electrostatic interactions.

Multivalency: the ability of a molecule to engage in multiple simultaneous interactions. In HMGB1, this is achieved through dynamic interactions between its structured domains and its IDRs.

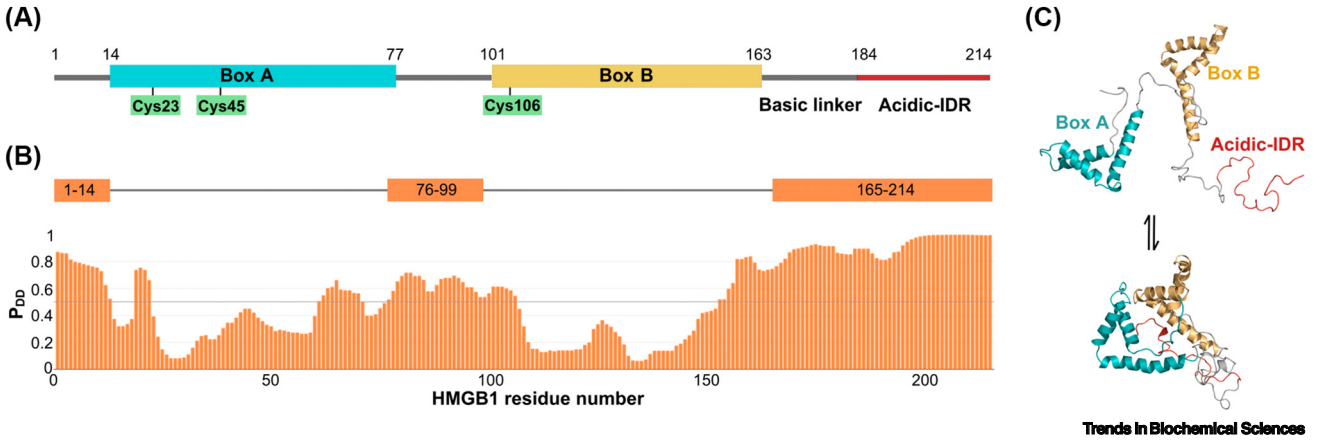

Figure 1. High mobility group box 1 (HMGB1) is a fuzzy protein. (A) Domain architecture of HMGB1. Boxes A and B are shown as cyan and wheat rectangles, respectively. The intrinsically disordered regions (IDRs) are represented by thin lines, and the basic linker and the acidic IDR (red) are explicitly labeled. Domain boundaries were defined based on the structure of HMGB1 (PDB: 2YRD). (B) IDR prediction (top) and disorder-to-disorder binding mode probability (P_{DD}) (bottom) estimated by the FuzPred webserver [83]. (C) Cartoon representation of HMGB1 in an open (top) and collapsed (bottom) conformations, with the same color coding as in (A).

The acidic IDR of HMGB1 is exclusively made up of D/E repeats and therefore is highly charged; indeed, two complementary papers show that it both causes autoinhibition of frHMGB1 and has chaperone-like activity [33,46]. The first study [46], based on fluorescence anisotropy, NMR experiments, and all-atom and coarse-grained MD simulations, provides evidence that the ensemble of relatively weak electrostatic interactions between the D/E repeats and the basic DNA-binding surfaces of the HMG boxes and the basic linker cause strong autoinhibition against binding to distorted DNA containing a cisplatin adduct. The autoinhibition equilibrium constant K_{ai} was estimated to be of the order of ~10–10^2 at physiological ionic strength: DNA binding by frHMGB1 was up to 100-fold stronger after removal of the acidic IDR [33]. The same authors showed that, although dynamic autoinhibition reduced the affinity to cisplatinated DNA, it increased the binding specificity and positively influenced the binding kinetics [46]. In detail, autoinhibition by the acidic IDRs accelerates target search and association via rapid diffusion in the presence of distracting decoys (i.e., non-functional high-affinity nucleic acids ligands) (Figure 2A). To achieve this acceleration, the protein must efficiently transition from an autoinhibited to an uninhibited conformation upon encountering the DNA target [46]. Conversely, removal of the acidic IDR enables stronger binding but increases the probability of being trapped by decoys, thus reducing the kinetics of binding to distorted DNA (Figure 2B). Later, using NMR, the same authors further showed that the D/E repeats and DNA have similar electrostatic properties and compete for the DNA-binding domains, and hypothesized an analogous mechanism for other DNA/RNA-binding proteins harboring D/E repeats [47]. In conclusion, the acidic IDR of frHMGB1 exemplifies how charged IDRs can finely tune chromatin-associated protein function by acting as dynamic autoinhibitory elements that balance DNA-binding strength, specificity, and kinetics through transient electrostatic interactions.

Fuzzy interactions between the acidic IDR and the histone tails

frHMGB1 engages in dynamic interactions not only with nucleic acids but also with other partners such as histones. Indeed, a pioneering paper showed that frHMGB1 is bound to chromatin through a network of fast dynamic interactions [48]. A decade ago, crosslinking experiments, gel filtration, and NMR spectroscopy showed that frHMGB1 binds to histone H3 via interactions between the acidic IDR of frHMGB1 and the H3 N-terminal tail [49]. Importantly, a 40 aa peptide, corresponding to the basic N-terminal tail of H3, was able to bind *in vitro* to the entire acidic IDR of frHMGB1 in a 1:1 complex, thereby competing with HMGB1

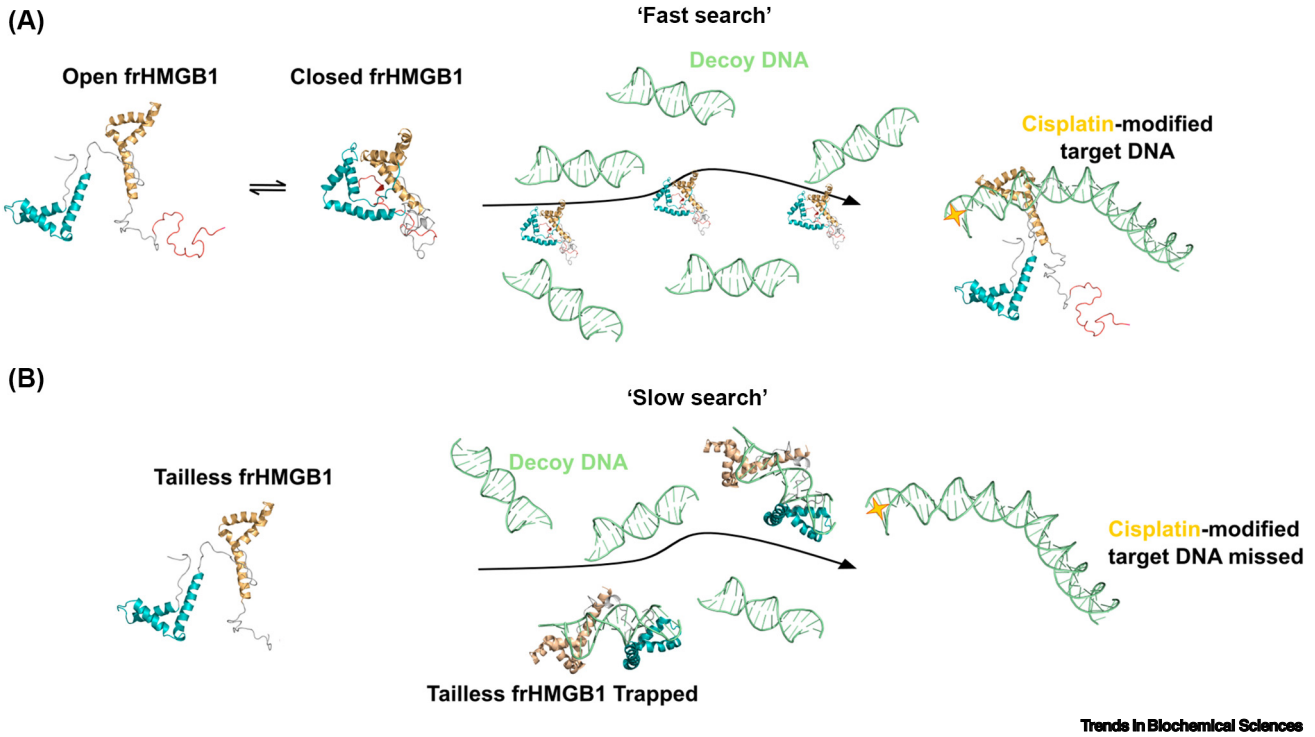

Figure 2. The autoinhibitory role of the acidic intrinsically disordered region (IDR) in fully reduced (fr) HMGB1–DNA interactions. (A) Autoinhibited closed conformation of frHMGB1 rapidly diffuses in the presence of distracting decoys DNA, accelerating the binding to distorted cisplatin-modified DNA. Upon encountering target DNA, frHMGB1 efficiently shifts from the closed to an open conformation. (B) Tail-less frHMGB1 results in inefficient target DNA recognition and is trapped by decoy DNA. Colors are as in Figure 1. DNA and cisplatin-modified DNA are shown in green and with a yellow star, respectively.

intramolecular interactions. Notably, CD and NMR studies showed that, upon binding, both the H3 N-terminal tail and the acidic IDR, although more constrained, maintain a high degree of flexibility without adopting any defined secondary structure. The authors proposed a working model in which the acidic IDR, once released upon binding of the HMG boxes to chromatin DNA, could establish fuzzy interactions with the H3 N-terminal tail, thus allowing frHMGB1 to localize near the nucleosome dyad. A combination of (i) disruption of H3–DNA interactions, and (ii) HMG box-induced untwisting of DNA was then proposed to promote DNA unwrapping and accessibility, thereby destabilizing the nucleosome core (Figure 3A) [49]. An alternative but not mutually exclusive interpretation is that the acidic IDR, upon interacting with the H3 tail, could be anchored to increase its local concentration and restrict the volume of exploration of the HMG boxes for bent or distorted DNA within the nucleosome; this is coherent with Bonaldi *et al.* [50] who showed that HMGB1 facilitates nucleosome relocation, a process that requires the formation of a DNA loop within nucleosomal DNA.

Competitive interactions also occur between the basic C-terminal tail of the linker histone H1 and the acidic IDR of frHMGB1 [51]. H1 and frHMGB1 occupancy on chromatin has been proposed to be mutually exclusive. It was thus hypothesized that interactions between them could promote displacement or replacement of one by the other (Figure 3B). Indeed, direct binding between H1 and frHMGB1 has been demonstrated *in vitro* by chemical crosslinking, gel filtration, and NMR analysis. Both H1 and frHMGB1 bind to linker DNA between nucleosomes but have opposing effects: H1 seals two turns of DNA around the octamer, whereas frHMGB1 destabilizes it, probably by bending the adjacent DNA. The interaction of the acidic IDR with the basic C-terminal tail of H1

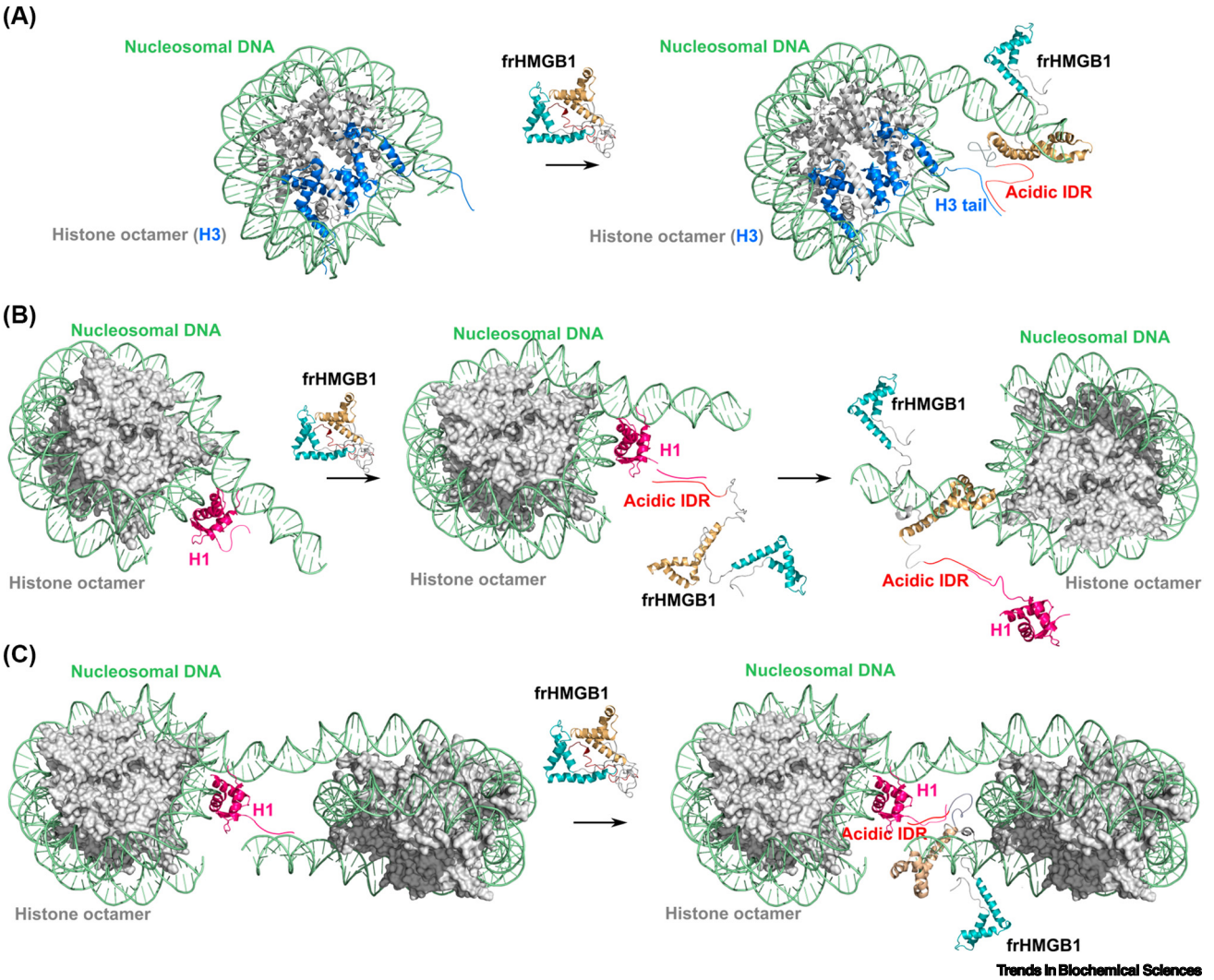

Figure 3. Fuzzy interactions of fully reduced (fr) HMGB1 with histone tails. (A) Model of fuzzy interactions with histone H3 (blue). The interaction between the frHMGB1 acidic intrinsically disordered region (IDR) and the basic N-terminal tail of histone H3, which is in proximity to the DNA entry/exit point, is proposed to promote nucleosomal DNA unwrapping. (B) Model of fuzzy interactions resulting in replacement of histone H1 (magenta). The interaction between the frHMGB1 acidic IDR with the basic C-terminal tail of H1 induces a conformational shift of frHMGB1 towards the open form, resulting in H1 detachment from DNA and its replacement by the open form of frHMGB1. (C) Model of fuzzy interactions between the frHMGB1 acidic IDR and the C-terminal tail of H1 resulting in co-occupancy of neighboring nucleosomes.

induces a conformational shift of frHMGB1 towards the open form, whereas the affinity of H1 for DNA is reduced, favoring its detachment and allowing its replacement by the open form of frHMGB1 (Figure 3B). It was thus proposed that this displacement/replacement mechanism in chromatin could facilitate nucleosome remodeling [51]. This model needs further support, and structural studies that can capture the transient nature of HMGB1 interaction with the entire nucleosome will be necessary to obtain a comprehensive mechanistic understanding of how HMGB1 counteracts the effects of linker histone H1 to increase nucleosome dynamics and DNA accessibility. Intriguingly, a recent cryo-electron microscopy (cryo-EM) study proposed that nucleosome co-occupancy by frHMGB1 and H1 contributes to the diversity of dynamic chromatin states (Figure 3C) [52].

The role of the acidic IDR in the chaperone-like activity of frHMGB1

HMGB1 abundance and dynamic interactions within the cell [53] make it an excellent candidate for chaperone activity of the adaptor/exchange type by transiently binding to one interactor and passing it over for interaction with another molecule [21,45,54]. This is the case for the transcriptional activator and tumor-suppressor p53 whose binding to its cognate DNA target site is facilitated by frHMGB1 [55] and tuned by the acidic IDR via its transient and differential shielding of both HMG boxes [56] (Figure 4). Crosslinking, gel filtration, and NMR analysis of different deletion mutants of both p53 and frHMGB1 have shown that the acidic IDR of frHMGB1 modulates the concerted mechanism through which boxes A and B act as protein and DNA chaperones, respectively, thereby promoting the binding of p53 to its cognate DNA. The distinct functional roles of the two HMG boxes appear to be finely regulated by the acidic IDR. At physiological ionic strength, box A is less tightly sequestered by the acidic IDR than box B [31] and is thus more readily available to act as a protein chaperone and recruit the transactivation domain 2 (TAD2) of p53. Conversely, box B, via its ability to bend DNA, provides an appropriate DNA structure to which p53 can easily bind. As it is often the case in short-lived fuzzy interactions, their strength can be subtly modulated by mass-balance effects, cellular conditions (e.g., pH and ionic strength), and post-translational modifications. As is typical of fuzzy interactions, the precise

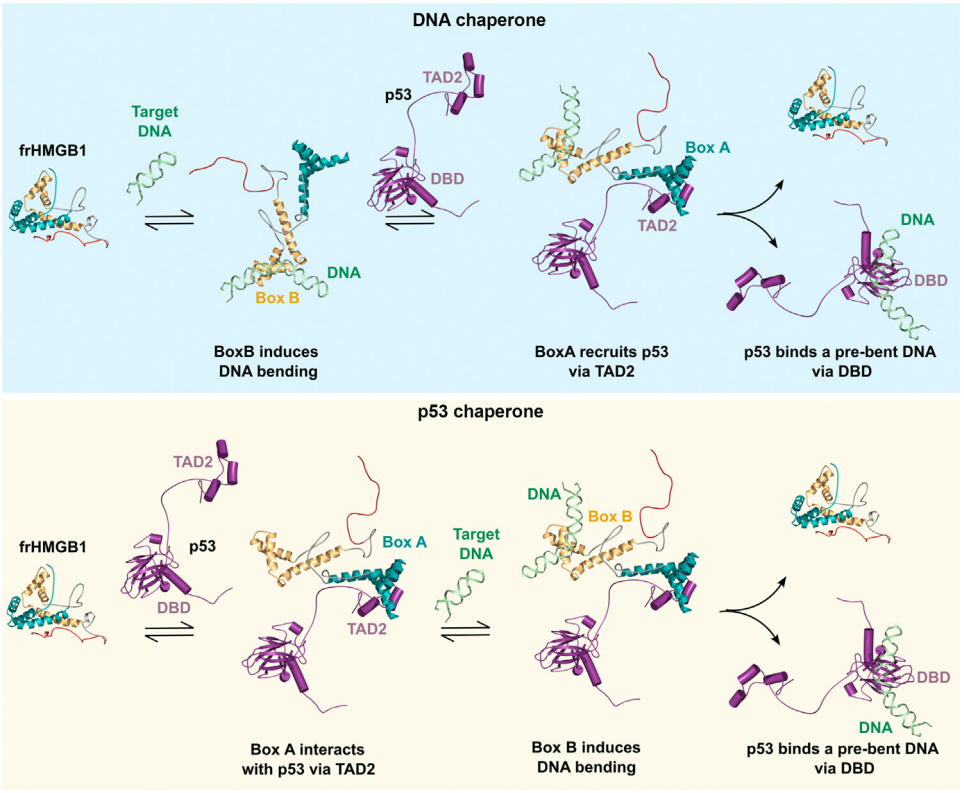

Figure 4. Fully reduced (fr) HMGB1 acts as a DNA and protein chaperone via its acidic intrinsically disordered region (IDR). (Top) frHMGB1 as DNA chaperone: box B has higher DNA-binding ability and bends DNA, facilitating DNA binding, whereas box A recruits p53 via the transactivation domain 2 (TAD2) and facilitates binding of the DNA-binding domain (DBD) to its target site. (Bottom) frHMGB1 as a p53 chaperone: box A is less tightly sequestered by the acidic IDR and can easily recruit the TAD2 of p53. Box B can then bind to and bend DNA, thus promoting the binding of the DBD to pre-bent DNA. Both mechanisms are modulated by the acidic IDR and result in facilitating the binding of p53 to its cognate DNA, ultimately resulting in transcriptional activation.

sequence of events leading to the facilitation of p53 binding is not well defined and not so relevant because all events involve an ensemble of equilibria. What is clear is that all the interactions in which frHMGB1 operates as a chromatin-remodeling factor or as protein/DNA chaperone would not be possible without being fuzzy – malleable, dynamic, and reversible.

The role of the acidic IDR in the ability of frHMGB1 to form condensates via liquid–liquid phase separation

Finally, the polyelectrolyte characteristics of the acidic IDR confer to frHMGB1 the ability to condense via **liquid–liquid phase separation (LLPS)** into dynamic, spherical liquid droplets both *in vitro* and *in vivo* [57]. Biomolecular condensates are membraneless organelles composed of higher-order, non-stoichiometric molecular assemblies containing proteins, RNA/DNA, and/or small molecules [58,59], and have emerged as important regulators of cellular responses [60]. LLPS is driven by multivalency or by multiple weak interactions that rapidly form, break, and reform [60], and HMGB1 achieves this multivalency through the dynamic interactions between the structured tandem domains and the acidic IDR. Replacement of the acidic IDR by an arginine-rich basic tail results in aberrant phase separation and nucleolar dysfunction, ultimately causing brachyphalangy, polydactyly, and tibial aplasia/hypoplasia syndrome, a complex human malformation syndrome [57]. However, whether the intrinsic ability of HMGB1 to generate condensates plays any role in cell physiology remains an open question.

The acidic IDR is a key driver of fuzzy interactions between HMGB1 and extracellular receptors

Once released into the extracellular milieu, HMGB1 in its different oxidation states functions as a potent proinflammatory mediator through a diverse network of interactions that rely both on HMGB1 post-translational modifications (such as reversible and terminal cysteine oxidation on boxes A and B) and conformational heterogeneity [1,2]. HMGB1 has several extracellular receptors, among which the Toll-like receptors 2 and 4 (TLR2 and TLR4), the receptor for advanced glycation end-products (RAGE), and CXCR4 stand out for their involvement in a wide array of inflammation-related processes. Although the role of the redox status of HMGB1 as a molecular switch that affects either the TLR4 or the CXCR4 axes is well established [61], the contribution of the acidic IDR to these interactions is in the early stage of investigation.

The acidic IDR modulates dsHMGB1 interactions with TLR4

A decade ago it became clear that dsHMGB1 was a potent activator of proinflammatory cytokine production via the binding and activation of TLR4 bound to its coreceptor myeloid differentiation protein 2 (TLR4•MD2) [26]; the other redox forms of HMGB1 are far less active. Notably, a recent study showed that dsHMGB1 undergoes the same intramolecular interactions as the reduced form and that tailless dsHMGB1 is a higher-affinity ligand for the TLR4•MD2 complex: surface plasmon resonance (SPR) experiments show a shift in affinity from 367 ± 19 nM for the full-length protein to 25 ± 1 nM for the tail-less form [62]. Because removal of the acidic IDR tail facilitates binding to TLR4, a reasonable question to ask is whether this removal occurs *in vivo*. Indeed, the same study has showed that it does [62]. dsHMGB1 binding to several neutrophil receptors, including TLR4, TLR2, and RAGE, promotes the formation of neutrophil extracellular traps (NETosis) [63], a process in which neutrophils extrude their own DNA decorated with histones, dsHMGB1, and elastase (a potent but low-specificity protease). The simultaneous binding of dsHMGB1 and elastase to DNA promotes the cleavage of dsHMGB1 between aa 170 and 180 within the basic IDR, causing the release of the acidic IDR and the potentiation of dsHMGB1 as a TLR4 ligand. Metformin, a biguanide derivative that functions as an anti-inflammatory and hypoglycemic drug, was shown through affinity purification using biotinylated metformin to directly bind to dsHMGB1 via its acidic IDR. Importantly, metformin also inhibited the TLR4-dependent

cytokine activity of dsHMGB1 both *in vitro* and *in vivo*, but was ineffective on dsHMGB1 lacking the acidic IDR. In fact, metformin appears to be the first inhibitor of HMGB1 (in all its redox forms) to target its acidic IDR [37].

frHMGB1 forms a fuzzy complex with CXCL12

Although accurate structural descriptions of the HMGB1–RAGE and dsHMGB1–TLR4•MD2 complexes are still lacking, our knowledge of the molecular details governing the interaction between frHMGB1, the chemokine CXCL12, and the GPCR CXCR4 has recently moved one substantial step forward [36]. More than a decade ago it was shown that frHMGB1 forms a physiologically relevant heterocomplex with CXCL12 (frHMGB1•CXCL12) that synergically promotes the recruitment of inflammatory cells to injured tissues via activation of CXCR4 [25,64]. Binding of frHMGB1•CXCL12 to CXCR4 induces receptor homodimer rearrangements and stimulates CXCR4-mediated signaling, resulting in increased ERK activation and calcium fluxes [25,64], and maintains CXCR4 on the plasma membrane in a β-arrestin 2-dependent manner [64]. Our understanding of how the synergistic action between frHMGB1 and CXCL12 occurs at the atomic level had been driven and, to some extent, biased by analogy to the rigid interaction mechanism that usually characterizes chemokine homophilic and heterophilic interactions [65]. NMR and computational studies primarily focused on the binding of CXCL12 to the structured parts of HMGB1, namely the isolated HMG boxes. This assumption resulted in a model in which HMGB1 binds two CXCL12 molecules that in turn interact with two CXCR4 receptors [25]. However, the role of the HMGB1 IDRs was totally ignored.

As is often the case for IDR and IDP interactions [17,66,67], an integrated strategy of solution methods (i.e., NMR, SAXS, ITC, microscale thermophoresis, and analytical ultracentrifugation) was necessary to describe the interface, affinity, stoichiometry, size, and shape of the heteromeric complex. A divide-and-conquer strategy was employed, utilizing full-length HMGB1 and tail-less HMGB1 (in both the disulfide and fully reduced forms), synthetic and recombinant acidic IDR peptides, and a locked monomeric form of CXCL12 to discriminate between heterophilic and homophilic CXCL12 interactions [36]. The results of this study shifted our understanding of this complex from classical rigid heterophilic chemokine dimerization to a fuzzy mechanism in which the acidic IDR plays a fundamental role in complex assembly. In contrast to previous suggestions, frHMGB1 binds to a single CXCL12 molecule, forming an equimolar, dynamic, interconverting ensemble of structures, each representing a different equilibrium state, rather than a singular, defined entity. This heterogeneity was confirmed by SAXS data which were best explained by multiple docking models generated through an ensemble optimization method: CXCL12 binds to frHMGB1 in a promiscuous manner that adapts to the many conformations of the acidic IDR and box A orientations (Figure 5). The acidic IDR does not have a single, fixed binding site, instead it presents a dynamic and diffuse 'binding cloud' [68] composed of multiple, similar binding sites while retaining considerable flexibility even in the bound state. A similar behavior has been recently observed for the complex between negatively charged and fully disordered prothymosin and the positively charged and folded globular domain of histone H1.0 [69].

We previously proposed an interaction model in which the acidic IDR acts as a 'wrapping antenna' that recruits the basic CXCL12 via long-range electrostatic interactions. The short lifetime of long-range electrostatic interactions allows CXCL12 to further interact with HMG boxes, particularly with the reduced form of box A, thereby competing with the autoinhibited conformation of frHMGB1. Of note, recent coarse-grained molecular dynamics simulations performed with an improved force field for disordered proteins were able to capture the complex interplay between the acidic IDR, box A, and CXCL12, in perfect agreement with our experimental data and the proposed interaction model [70]. Importantly, the N-terminus of bound CXCL12 is not

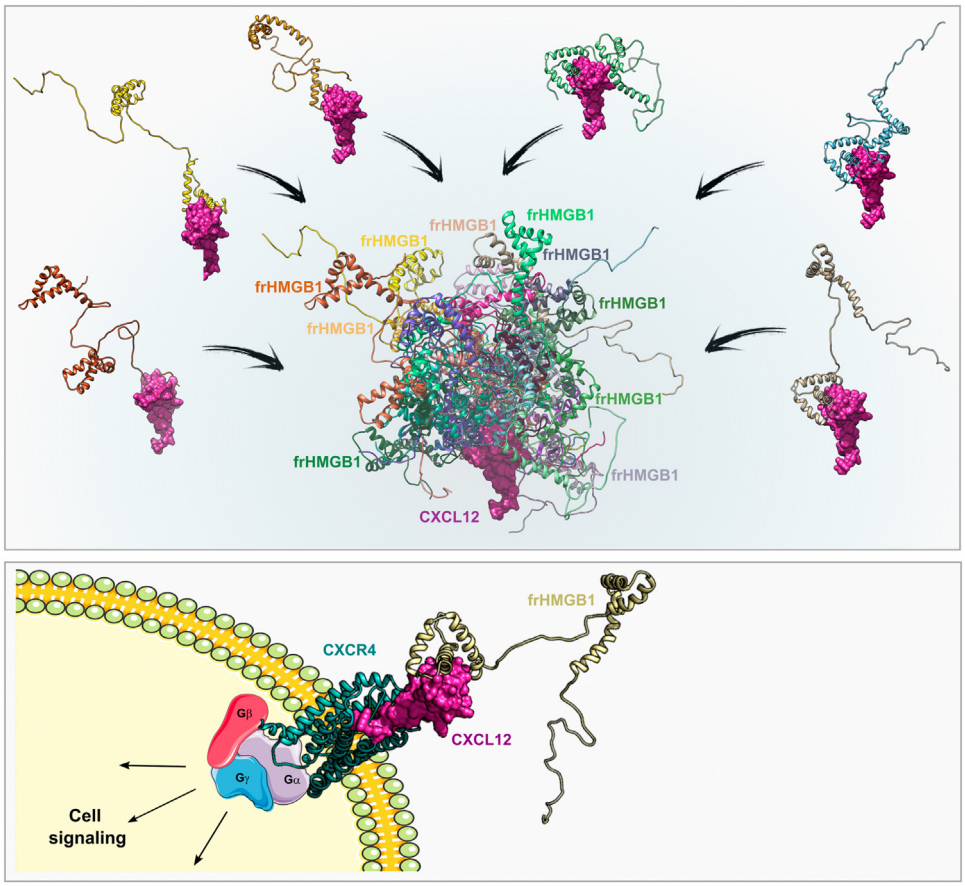

Figure 5. Fully reduced (fr) HMGB1 forms a fuzzy complex with CXCL12. (Top) CXCL12 (magenta) binds to HMGB1 in a promiscuous manner by adapting to the many conformations of the acidic intrinsically disordered region (IDR) and orientations of box A. The differently colored structures derive from *small-angle X-ray scattering* (SAXS) ensemble optimization analysis [36]. The superposition of these conformers best represents the dynamic and diffuse nature of the frHMGB1•CXCL12 binding cloud. (Bottom) Schematic representation of the interaction of frHMGB1•CXCL12 and CXCR4 that promotes receptor conformational rearrangements and modulates downstream pathways.

involved in the interaction with frHMGB1, as assessed by NMR experiments. It can therefore insert into the cradle formed by the transmembrane helices of CXCR4 to form a ternary complex (Figure 5). Importantly, the existence of frHMGB1•CXCL12•CXCR4 ternary complexes on the cell surface was supported by proximity ligation assay experiments on malignant mesothelioma cells [36]. The acidic IDR facilitates the binding of the frHMGB1•CXCL12 heterocomplex to CXCR4, implying that the acidic IDR also plays a major role of in the formation of the frHMGB1•CXCL12•CXCR4 ternary complex.

The current data do not indicate whether a specific subset of conformations of the frHMGB1•CXCL12 heterocomplex preferentially bind to CXCR4, thereby promoting specific receptor conformational rearrangements and related downstream pathways. In fact, the ternary complex may still retain the fuzziness of the frHMGB1•CXCL12 heterocomplex. Integrative biophysical and biochemical approaches, coupled to classical computational and artificial intelligence methods tailored to IDRs [70–73], will be necessary to faithfully capture the dynamics of the ternary complex.

Concluding remarks

The concept of fuzzy interactions provides a new perspective for understanding the extraordinary functional diversity of HMGB1. Over the years evidence has accumulated that HMGB1 is intrinsically fuzzy and engages in the formation of fuzzy complexes. Fuzziness is primarily conferred by its acidic IDR, and this explains the extreme evolutionary conservation of its simple repetitive sequence. The acidic IDR plays a key role in modulating the interactions of HMGB1 both within the nucleus, particularly with DNA, and extracellularly with its main receptors TLR4 and CXCR4. This suggests that the acidic IDR likely regulates most, if not all, of the interactions HMGB1 is involved in, including its engagement with other extracellular receptors and its cytoplasmic interaction with beclin. The same chemical entity, the acidic IDR, can perform distinct functional roles and can act either as an autoinhibitory element or as a molecular chaperone, thereby expanding the functional repertoire of HMGB1. Autoinhibition and chaperoning are inseparable, and often work synergistically, for example by enhancing the ability of HMGB1 to search for binding sites on DNA [46]. Notably, at least one known pathophysiological mechanism can lead to the removal of the acidic IDR, thus modulating the activity of HMGB1.

From a translational point of view, the multifaceted binding modes of HMGB1 in all its redox forms represent a particularly intriguing and challenging biomolecular target [74] in which not only structured [75–78] but also disordered parts could be exploited [37], thereby enlarging our opportunities for therapeutic intervention [79]. Future research on the molecular underpinnings of HMGB1 interactions, including advances in structural, computational, and chemical biology as well as in artificial intelligence, are likely to uncover novel strategies to modulate its activity in health and disease (see Outstanding questions). Ultimately, the example of HMGB1 highlights the broader significance of dynamic molecular association and fuzziness in mediating complex biological functions and their targeting. A growing number of studies provide examples of dynamic, charge-mediated interactions that give rise to multi-state ensembles. These studies range from ultrahigh-affinity complexes that retain intrinsic disorder, such as the interaction between linker histone H1.0 and prothymosin α [17], to dynamic multivalent interactions that are key to enzymatic regulation and substrate recruitment, particularly in kinases and phosphatases [80]. The exploration of novel regulatory mechanisms involving fuzzy interactions, as well as their potential for pharmacological targeting, is still in its early stages [81,82] and represents a promising frontier in drug discovery.

Outstanding questions

How do HMGB1 interactions with H3 and H1, or isotypes or modifications of these molecules, control chromatin dynamics? Does HMGB1 define or control specific chromatin conformations or nuclear domains?

What is the role of the intrinsic ability of HMGB1 to undergo LLPS in normal cell physiology or under stress conditions?

Does the frHMGB1•CXCL12•CXCR4 ternary complex itself retain the fuzzy nature of the binary HMGB1•CXCL12 heterocomplex?

Does a particular subset of conformations within the dynamic ensemble of the HMGB1•CXCL12 heterocomplex preferentially bind to CXCR4, and if so, how does this influence receptor conformational rearrangements and downstream signaling pathways?

How do the IDRs of HMGB1 influence its binding to other receptors or proteins (e.g., RAGE and beclin-1)?

Do post-translational modifications (e.g., acetylation, lactylation, phosphorylation, and methylation) influence the conformational dynamics of HMGB1?

Beyond its cleavage by neutrophil elastase, are there other specific pathophysiological mechanisms that lead to the removal or modification of the acidic IDR of HMGB1 *in vivo*?

Is the acidic IDR a cellular physicochemical sensor, and do the inter- and intramolecular fuzzy interactions respond to physiological changes in the environment?

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Declaration of interests

M.E.B. is founder and part-owner of HMGBiotech, a company that provides goods and services related to HMGB proteins. The other authors declare no competing interests.

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