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The next wave of interactomics: Mapping the SLiM-based interactions of the intrinsically disordered proteome

Norman E. Davey[1], Leandro Simonetti[2] and Ylva Ivarsson[2]

Abstract

Short linear motifs (SLiMs) are a unique and ubiquitous class of protein interaction modules that perform key regulatory functions and drive dynamic complex formation. For decades, interactions mediated by SLiMs have accumulated through detailed low-throughput experiments. Recent methodological advances have opened this previously underexplored area of the human interactome to high-throughput protein–protein interaction discovery. In this article, we discuss that SLiM-based interactions represent a significant blind spot in the current interactomics data, introduce the key methods that are illuminating the elusive SLiM-mediated interactome of the human cell on a large scale, and discuss the implications for the field.

Addresses

[1] Division of Cancer Biology, The Institute of Cancer Research, 237 Fulham Road, London, SW3 6JB, UK

[2] Department of Chemistry - BMC, Uppsala University, Box 576, Husargatan 3, 751 23, Uppsala, Sweden

Corresponding authors: Ivarsson, Ylva (ylva.ivarsson@kemi.uu.se); Davey, Norman E. (norman.davey@icr.ac.uk)
(Davey N.E.), (Ivarsson Y.)

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Keywords

Short linear motifs (SLiMs), Protein–protein interactions, Interactomics, High-throughput methods, Protein–peptide interactions.

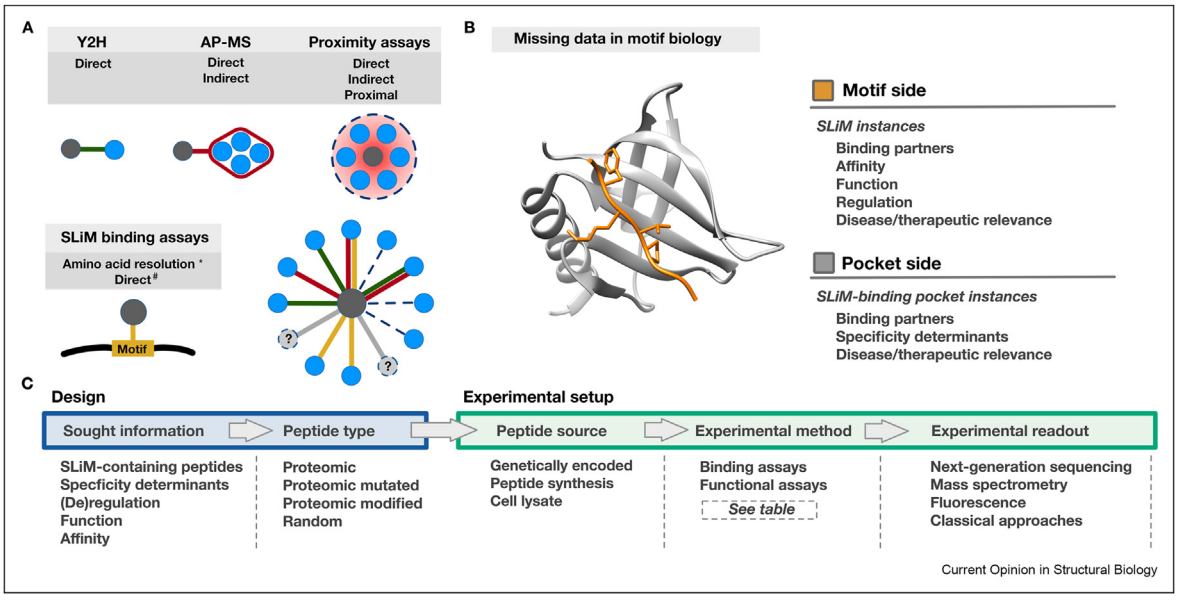
Introduction

Over the last decade, numerous breakthroughs in the scale and quality of interactomics methods have reshaped our understanding of the human interactome. This era of high-throughput interactomics has largely been driven by technical advances that have enabled large-scale mapping of protein interactions. Three complementary yet distinct approaches are central to the interactomics data explosion: yeast two-hybrid (Y2H) discovering direct binary interactions [1]; affinity purification coupled to mass spectrometry (AP-MS) discovering protein complexes [2]; and proximity labeling mass spectrometry (e.g. BioID [3], TurboID [4], Apex2 [5]) defining cellular proximity [6] (Figure 1a). Each of these approaches has provided a map of the human interactome. When integrated, in conjunction with other sources of data on protein–protein interactions (PPIs), they result in a draft map of the wiring of the interactome of the human cell [7,8].

Despite the revolutionary contribution of interactomics data to the understanding of cell biology, the current interactome is biased towards PPIs that are most readily captured by widely applied methods [1,2,6]. The biases result from technical limitations of the method used, such as the trade-off between noise and signal in AP-MS, which can result in a bias towards stable interactions, and the Y2H requirement for proteins that are not toxic in yeast, can translocate to the nucleus and do not autoactivate in the absence of an interaction. Other factors relate to interactions that occur conditionally (e.g. dependent on post-translational modifications; PTMs), in specific cell types, or between low-abundance proteins. Consequently, many subsets of the interactome remain undersampled. Also, the classical definition of a PPI has somewhat drifted as many of the reported interactions are not through direct binary interfaces but report on larger complexes or on protein proximity (Figure 1a). Recent advances in deep learning for protein complex modeling may successfully add interface-level information and improve our confidence for a direct interaction in a single stroke [9]. However, given the lack of data for the inaccessible subsets of the interactome, the blind spots for the interactomics approaches may be reflected by the machine learning approaches.

One of the main blind spots of the current interactome is short linear motif (SLiM)-mediated interactions as they are often of low affinity and transient [10]. SLiMs are a distinct class of interaction modules generally

Figure 1



(**a**) Schematics of the interaction types returned by commonly used high-throughput PPI detection methods. The network illustrates that the approaches provide complementary data. The # indicates that most SLiM-binding assays discussed (e.g., arrays, display methods, and affinity measurements) are providing information on direct binary interactions, but when combined with MS read outs (e.g., PRISMA and hold up) can report on both direct and indirect interactions. The * indicates that while most SLiM discovery methods can be geared towards the identification of binding sites with amino acid resolution (e.g. by dense tiling of proteins by overlapping peptides, or by mutagenic analysis), this is not always the case. (**b**) List of important information required for the comprehensive characterization of a SLiM instance or SLiM-binding pocket. Representative structure of a SLiM-mediated interaction showing the SLiM-binding pocket of the serine/threonine-protein phosphatase 4 regulatory subunit 3A (PP4R3A) EVH1 domain bound to a model FxxP motif-containing peptide (PDB ID:6R8I) [31]. (**c**) Design principles for a high-throughput SLiM analysis method.

found in intrinsically disordered regions of the proteome. SLiMs are compact (<10 amino acids in length) with simple specificity determinants (~3–4 key residues define the majority of the specificity and affinity of binding) [11]. They regulate key processes in the cell by acting as targeting signals to control protein localization, transactivation domains to modulate transcription, enzyme docking sites to define substrates for PTMs, degrons to modulate protein stability, and binding sites to drive dynamic complex formation [12]. Most of the currently known SLiM-based interactions were characterized by low-throughput studies [13], as the often low-to-mid μM affinity range of SLiM-mediated interactions makes them relatively difficult to capture using the PPI discovery approaches mentioned above. The lack of methods to specifically discover and characterize transient, low-affinity SLiM-mediated interactions in a high-throughput manner was obvious (Figure 1b).

In recent years, the SLiM field has made a concerted effort to develop large-scale approaches specifically designed to characterize SLiMs (Table 1). The resulting methods encompass a set of approaches that assay multiple peptides in a single experiment with the goal of testing the binding or function of SLiMs. The applications include discovering SLiM-containing peptides, defining the specificity determinants of SLiMs, understanding the effects of specific mutations in a SLiM, quantifying the affinities of SLiM-mediated interactions, or functionally characterizing SLiM-containing peptides. Some approaches can perform several of these applications in a single experiment, however, usually with a trade-off to quality and scale. The assayed peptides can be designed based on randomized sequences, protein-derived sequences, or mutated protein-derived sequences. The number of peptides tested can range widely in scale from tens of peptides to millions of peptides. When used for PPI discovery, the peptide-centric nature of these methods allows the discovery of SLiMs with amino acid resolution.

In this article, we introduce experimental SLiM discovery tools focusing on recent approaches and studies that have pushed the SLiM discovery field into the high-throughput era. The methods are divided into two major classes: *binding assays* that analyze the physical interaction between the peptide and peptide binding partner, and *functional assays* that measure the downstream function encoded by the motif (Table 1). We briefly discuss the importance of applying complementary approaches to confidently map the

Table 1

Overview of methods for large-scale identification and characterization of SLiM-based interactions and SLiM-mediated functions. Semi-quantitative read outs indicate that the read out of the assay is related to the relative binding strengths of interactions probed (e.g. denser spot intensities for higher affinity interactions).

Type	Method	Scale of peptides published	Advantages	Challenges	Preys	Bait avidity to increase capture	PTM-dependent interactions described	Recent publications
Binding assays								
Synthesized peptide arrays	Peptide arrays	100s	Semi-quantitative readout. Ease of experiment. Ease of studying effects of PTMs.	Loss of low-affinity interactions. Limited by cost of arrays. One protein at a time.	Purified proteins	Yes	Yes	[17,18]
	PRISMA	100s	Discovery of SLiM-binding proteins. Ease of studying effects of PTMs.	Loss of low-affinity interactions. Difficult to find interactions with low-abundant proteins. Interactions found may be indirect rather than direct. Limited by MS access. Noisy data.	Cell lysate	Yes	Yes	[20,21,23]
Display methods	ProP-PD	1,000,000	Scalability (10^{11} variants). Highly parallelizable. Multiplexed NGS analysis.	Library size is limited by the cost of designed oligonucleotide library. Multiple rounds of selections are required to enrich binders. For multiple parallel screens – bait protein production	Purified proteins	Yes/No[a]	Yes[b]	[26,29]
	Yeast-surface display	11,000	Scalability (10^{8} variants). Information on binders and non-binders. Library-on-library screens are possible.	Limited by FACS sorting Limited in terms of parallelization opportunities	Purified proteins	Yes	No	[35–37]
	Bacterial-surface display	420,000	Scalability (10^{11} variants). Information on binders and non-binders.	Library size is limited by the cost of designed oligonucleotide library. Screening is limited by FACS sorting. Limited in terms of parallelization opportunities	Purified proteins	Yes	Yes	[41,42,72]

(*continued on next page*)

Table 1. (*continued*)

Type	Method	Scale of peptides published	Advantages	Challenges	Preys	Bait avidity to increase capture	PTM-dependent interactions described	Recent publications
	mRNA display	10,000	Large peptide libraries possible (10^{13}). Cell free.	Library size is limited by the cost of designed oligonucleotide library. Multiple rounds of selections are required to enrich binders.	Purified proteins	No	No	[46]
Quantitative binding assays	MRBLE-pep	100	Multiplexed affinity determinations.	Limited by synthesis of peptide-linked beads Requires specialized equipment.	Purified proteins	Yes	Yes	[47]
	Hold-up assay	500	Affinity determinations. Many options for detection. Versatile – allows peptide or protein as bait.	Peptide synthesis if using peptide bait. Bait protein production if using purified proteins. Limited by peptide synthesis and detection cost.	Purified proteins Cell extract	Yes	Yes	[51,52]
In cell binding assays	Pheromone signaling competitive growth assay	1000	Binding under physiological conditions. Affinity ranked list of peptides.	In cell assays can introduce complex biases. Limited by transduction of cells during library construction. NGS costs.	Genetically encoded	No	No	[58]
	MAPK competitive growth assay	10,000	Binding under physiological conditions. Affinity ranked list of peptides.	In cell assays can introduce complex biases. Limited to the MAPK kinase studied. Limited by transduction of cells during library construction.	Genetically encoded	No	No	[59]
Functional assays	Transactivation assays	10,000	Functional information. Semi-quantitative readout.	No information on binding partner. Limited by transduction of cells during library construction and/or FACS sorting	N/A	No	No	[46,62]
	Degradation assays	50,000	Functional information. Semi-quantitative readout.	No information on binding partner. Limited by transduction of cells during library construction and/or FACS sorting	N/A	No	No	[64,65,67,71]

[a] Monovalent display on P3, multivalent display on P8.

[b] Using targeted library design (e.g. using phosphomimetic mutations) or enzymatic treatment of libraries. Note that similar strategies can be applied to different display techniques.

nsient SLiM-based interactions and the significance of these approaches to the next wave of interactomics.

In vitro analysis of SLiM-based interactions

Qualitative *in vitro* binding assays

Qualitative screening (or selection) assays are used to find SLiMs among a large number of peptides and can be grouped into array and display methods.

Peptide arrays

A peptide array is a set of peptides immobilized on a solid support (e.g. glass or a cellulose membrane) that is probed for binding to a protein of interest.

Classical peptide arrays

Peptide arrays have been applied for specificity determinant screening using substitution scanning mutagenesis, for scanning a tiled protein for binding regions, or for larger-scale motif discovery using proteomic peptides. Peptide arrays are commonly used to study binding and binding determinants of individual proteins through the incubation of an array with a purified protein. The interaction with the protein of interest is then detected using antibodies, chemiluminescence, or autoradiography. High-density peptide arrays can present hundreds of thousands of peptides [14,15]; however, for most SLiM discovery applications, the number has been in the range of hundreds of peptides [16–18]. Peptide arrays were, for example, used to explore the molecular determinants of the ATG8-binding LIR motifs [17] and the ubiquitin-binding DisUBM motif [19].

Protein interaction screen on peptide matrix (PRISMA)

Peptide array protocols have been extended by including AP-MS identification of the bound protein(s), which allow exploratory analyses to discover novel SLiM-binding partners from cell lysate (Table 1). In particular, protein interaction screen on peptide matrix (PRISMA) has been developed for the discovery of SLiM-binding partners [20,21]. While allowing de novo discovery of SLiM-mediated interactions, the results appear noisy and require careful data analysis [22]. A particularly interesting application of this approach is the analysis of disease mutations in the intrinsically disordered regions of the human proteome to analyze the gain and loss of SLiM-mediated interactions linked to disease [23]. Recently, it was also applied to find interactions of microprotein potentially encoded by small open reading frames [24].

Display methods

Display methods genetically encode the sequence of the peptide such that enrichment of the displayed peptide, through binding to a protein of interest, results in the enrichment of the coding sequence. This coding sequence can then be identified and quantified using next-generation sequencing (NGS).

Proteomic peptide phage display

In proteomic peptide phage display (ProP-PD) [25], libraries of protein-derived peptides are multivalently (P8) or monovalently (P3) presented on the surface of the filamentous M13 phage [25–27]. The libraries are used in selections against immobilized purified bait proteins, followed by NGS analysis. Libraries displaying peptides from yeast, human, and viral proteomes have been developed, and over 100 protein domains have been screened against these libraries providing information on thousands of interactions [25,26,28–33]. ProP-PD has further been applied to the identification of phospho-modulated interactions [33].

Yeast-surface display

In yeast-surface display, peptides are displayed on a yeast cell surface protein (typically Aga2p) [34]. A library of peptides is multivalently displayed on yeast cells and incubated with a fluorescently labeled bait protein (or a protein with an antibody-binding epitope). Cells displaying binding peptides are then sorted and analyzed by sequencing. Yeast-surface display was recently applied to screen for SARS-CoV-2 peptides binding to the major histocompatibility complex proteins [35]. The approach can be tuned to determine relative affinities [36], and variants of the approach have been developed for library-on-library affinity characterization of SLiM-based interactions [37].

Bacterial display

Bacterial display assays are similar to the experimental setup of yeast display, but the peptides are linked to bacterial outer membrane proteins for multivalent display [38,39]. Using a library composed of single and double mutants of TRAF2/3/5-binding peptides, it was shown that the NGS results after sorting can generate quantitative binding information on variant peptides [40]. The approach, termed MassTitr bacterial display, was recently scaled to proteome-wide screening by tiling the human proteome as 36 amino acid peptides and applied to find interactors of the ENAH EVH1 domain [41,42].

mRNA display

mRNA display screens peptide-RNA fusions in a cell-free format. In this case, *in vitro* translated peptides are covalently linked to their encoding mRNA via a puromycin linkage [43]. The peptides are used in selections against an immobilized bait, followed by reverse transcription and sequencing. The approach is often used to find high-affinity ligands with inhibitory potential [43] and can provide high-throughput quantification of binding kinetics [44]. mRNA display has been applied to find endogenous SLiM-based interactions, as showcased by the search of KEAP1 ligands using a library of fragmented mRNAs from human cells [45], and for evaluating transactivation domains (TAD) interactions with MED15 [46].

Quantitative binding assays

A distinct class of *in vitro* methods exists where the focus of the binding assay is to produce accurate quantitative data for a large number of protein–peptide pairs.

MRBLE-pep

MRBLE-pep assays peptides synthesized on spectrally encoded lanthanide beads to perform medium-throughput quantitative measurement of protein–peptide interactions [47]. The peptide-bead library is pooled and incubated with a fluorescently labeled protein of interest. The sample is then imaged to quantify binding and identify the peptide-bead pairs. Measurements are repeated at distinct concentrations to create concentration-dependent binding curves. The approach has been used to determine affinities for peptides binding to the PP2A-B56 and calcineurin phosphatases [47,48].

Hold-up assay

SLiM-based hold-up assays are comparative retention assays where peptides are immobilized on beads, the beads are distributed in a filter plate, proteins of interest are allowed to bind at equilibrium, and the unbound proteins are collected by filtration. The quantity of a protein in the total and unbound fractions is determined using capillary electrophoresis, intrinsic fluorescence, or MS. The results are then correlated with dissociation constants determined through classical affinity measurements [49–52]. The approach has been applied to characterize the affinities of 65,000 interactions involving PDZ domains and PDZ-binding motifs [51], explore the effects on PTMs on the binding landscape [53,54], and was recently developed for the determination of affinities with proteins in cell lysate [52].

In cell binding assays

Several in cell binding assays have been applied to screen for SLiM binding in cellular settings, including Y2H variants [55–57] and recent competitive growth assays that link peptide binding to cell proliferation.

Pheromone signaling competitive growth assay

A yeast pheromone signaling-based assay was developed to explore the specificity of LP motif docking interactions of the yeast G1 cyclin Cln2 using deep mutational scanning. Cell growth was encoded in an interaction between a chimeric signaling protein $Ste20^{Ste5PM}$ and the Cln2, whereby a functional LP docking motif can recruit Cln2, leading to phosphorylation of the $Ste20^{Ste5PM}$ reporter thereby blocking the signaling that inhibits cell division in response to pheromone [58]. The change in the relative abundance of a library of peptides in a cell population is calculated at different timepoints and analyzed to provide relative quantification of the affinities of the LP motif interactions.

MAPK competitive growth assay

A yeast growth assay has been developed leveraging the toxicity of exogenous mitogen-activated protein kinase (MAPK) signaling components to elucidate MAPK docking motifs [59]. The assay exploits the fact that human MAPKs p38α/JNK1 can be activated by MKK6 and active p38α/JNK1 results in growth suppression in yeast cells lacking endogenous MAPK Hog1 and MKK Pbs2. As the interaction between the MAPK and MKK is dependent on a single docking motif and linked to cell growth, the manipulation of the interaction allows for systematic exploration of MAPK kinase docking motifs. The reporter of the system is a third component, namely glutathione transferase-fused peptides to compete with the MAPK-substrate docking interaction, thereby rescuing cell growth.

Functional assays

Some classes of motif are amenable to high-throughput analysis using functional assays, which identify the consequences of SLiM-mediated interactions, but do not directly report on an interaction or binding partners (Table 1).

Transactivation assays

Several functional studies have focused on the discovery of TADs, a class of SLiMs which are bound by transcriptional coregulators and regulate transcription. The read out of TAD assays is generally cell viability [60] or the production of a fluorescent reporter (e.g. GFP) as a function of transcriptional activity [46,61,62]. NGS analysis reveals the identity of the TAD peptides in the population of cells with functional TAD interactions. The specificity determinants of TAD sequences have been defined using libraries of randomized peptides and through single and double mutant analysis of previously known TADs [60,61]. Designed transcription factor-derived peptides have been used to discover yeast TADs [46], human TADs [62], and motifs involved in transcriptional repressions [63].

Degradation assays

Large-scale functional assays have provided novel insights into degradation motifs, or degrons, a class of SLiMs that promote protein degradation. In these assays, a putative degron is fused to a fluorescent protein reporter. The reporter is co-transcribed with a second control fluorescent reporter that is translated as a separate protein. Functional degrons lead to the degradation of the fluorescent reporter relative to the control fluorescent reporter, and cells can be sorted by FACS into bins, allowing peptides that drive reporter degradation to be quantified by NGS. Stability profiling assays have resulted in the discovery of numerous novel N- and C-terminal degrons [64–67].

Discussion

We briefly surveyed methods that are advancing the SLiM discovery field into the high-throughput era. Each method can be applied to answer distinct questions (qualitative, quantitative, and functional). The display methods are very powerful in terms of library sizes and are key methods for expanding the SLiM-mediated interactome. The PRISMA assay (and the hold-up assay if coupled to MS) allows the identification of the binding partners of SLiM-containing peptides from cell lysate. Among the quantitative binding assays, the hold-up assay is highly promising given the ability of the method to generate quantitative data on a large scale. The functional assays provide complementary information to the binding assays, however, with the caveat that they are often dedicated to the discovery of a specific type of SLiM and they do not provide a direct readout of binding. Each method thus provides complementary information.

Given the largely unexplored search space of SLiM-based interactions, there is a need to combine the different approaches. Indeed, as for other interactomics data, the identification of an interaction by multiple methods increases confidence, so the generation and integration of complementary approaches will be key to defining a comprehensive map of the SLiM-mediated interactome. In addition to the SLiM discovery methods outlined, other interactomics approaches and computational searches for SLiMs will continue to contribute to the SLiM discovery field as described elsewhere [68,69].

However, difficulties still exist, such as the challenge of discriminating which of the identified *in vitro* biophysical binding events occur in a cellular setting and have functional consequences. Currently, there is a need for extensive downstream validation of the functionality of the newly discovered motifs in the context of full-length proteins and under relevant cellular conditions. Diverse functional assays leveraging the same set of underlying design principles and technologies (Box 1), providing high-throughput functional validation would accelerate this process. Furthermore, the application of base and prime editing technology [70] to mutate and insert SLiMs *in situ* will likely simplify *in vivo* functional assays. Other challenges relate to finding conditional PTM-dependent interactions. Although not discussed here, several of the methods described (e.g. peptide arrays, hold-up) can be used also for finding PTM-dependent interactions.

This era of high-throughput SLiM-based interactomics data has already begun and will reveal exciting insights into the wiring of the cell, the deregulation of the cell by disease mutations, and open new avenues for therapeutic intervention. Almost a decade ago, Tompa et al. predicted one hundred thousand SLiM-mediated interactions remain undiscovered in the human interactome [10]. The tools are now in place to test this prediction and fully characterize this elusive part of the human proteome. We expect SLiM discovery approaches to result in the unprecedented generation and release of information over the coming years.

Box 1. Reusing old parts to make new tools

Many major scientific breakthroughs are incrementally driven by technical advances that result in the availability of novel methods or facilitate a transition from low throughput to high throughput. In the SLiM discovery field, the transition to a large-scale discovery era was built on such advances, namely the ability to (i) encode/synthesize and (ii) quantify large numbers of peptides accurately and at a relatively low cost. At the core of every high-throughput SLiM screen is a library of peptides under investigation. There are two major sources of these peptides: peptide synthesis and oligonucleotide synthesis. In both cases, the scale, cost, and quality of synthesis have improved continuously over the past decade. The technology to quantify the experimental readout has mirrored the advance to the peptide encoding. Three technologies, in particular, are widely used in the available SLiM analysis methods: next-generation sequencing (NGS); fluorescence-activated cell sorting (FACS); and mass spectrometry (MS). The blueprint for high-throughput methods for the analysis of SLiM-mediated interactions is now clearly established (Figure 1c). Both binding and functional assays have taken advantage of the simplicity and transplantable nature of SLiMs. They encode a library of peptides of interest in a system that can separate motif-containing from non-motif-containing peptides based on either functional or biophysical discrimination and quantify that discriminatory attribute using well-established experimental readouts. Huge diversity and innovation are possible using these simple experimental design principles, and much of the possibilities in this space remain to be explored. Consequently, the methods developed to date are likely to be joined, particularly in the functional assay space, by further methods in the coming years.

Declaration of competing interest

Nothing declared.

Data availability

The review is based on published papers.

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** of outstanding interest

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