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Mapping of protein- and chromatin-interactions at the nuclear lamina

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Abbreviations: ChEC, chromatin endogenous cleavage; ChIC, chromatin immunocleavage; FPLD, Dunnigan familial partial lipodystrophy; HGPS, Hutchinson-Gilford Progeria syndrome, INM, inner nuclear membrane; MS, mass spectrometry; NE, nuclear envelope; NET, nuclear envelope transmembrane protein; NPC, nuclear pore complex; ONM, outer nuclear membrane; OST, OneSTrEP; PCP, protein correlation profiling; Srebp1, adipocyte differentiation factor sterol element binding protein 1; Y2H, yeast two-hybrid

The nuclear envelope and the lamina define the nuclear periphery and are implicated in many nuclear processes including chromatin organization, transcription and DNA replication. Mutations in lamin A proteins, major components of the lamina, interfere with these functions and cause a set of phenotypically diverse diseases referred to as laminopathies. The phenotypic diversity of laminopathies is thought to be the result of alterations in specific protein- and chromatin interactions due to lamin A mutations. Systematic identification of lamin A-protein and -chromatin interactions will be critical to uncover the molecular etiology of laminopathies. Here we summarize and critically discuss recent technology to analyze lamina-protein and-chromatin interactions.

Introduction

The nuclear periphery is marked by the nuclear envelope (NE), which is composed of an outer and inner nuclear membrane (ONM, INM, respectively) interrupted by nuclear pore complexes (NPC).[1] The nuclear lamina lines the NE and consists of a large collection of proteins, most prominently the intermediate filament A-type (Lamin A, C) and B-type lamins (Lamin B1, B2), INM proteins anchoring the lamina to the NE (including Emerin, MAN1, LAP2, Nesprin) and proteins that modulate and interact with chromatin such as BAF, HP1 and histone deacetylase 3 (**Fig. 1**).[2]

Mutations in the lamina's major constituent, particularly the A-type lamins, cause a diverse set of human diseases collectively referred to as laminopathies; these include several types of muscular dystrophies, lipodystrophies, cardiomyopathies, neurological disorders and premature aging syndromes.[3] The phenotypic diversity of laminopathies is hypothesized to be caused by lamin A mutations affecting specific lamina-protein and -chromatin-interactions, thereby compromising nuclear integrity, higher-order chromatin organization, gene expression and/or various other nuclear processes.[4] In line with this notion are the observations that Dunnigan familial partial lipodystrophy (FPLD)-associated lamin A mutations specifically disrupt interaction with the adipogenic transcription factor *Srebp1*[5] and that failure of progerin, a lamin A mutant that causes premature aging, to interact with the NURD chromatin remodeling complex contributes to loss of peripheral heterochromatin, a hallmark of the premature aging disorder Hutchinson-Gilford Progeria syndrome (HGPS).[6] Another progeria-associated lamin A mutation, E145K, leads to aberrant interaction of the lamina with telomeres.[7]

A key challenge in the field of lamin biology is to identify all protein and chromatin interactions at the nuclear periphery. Over the past years, several approaches have been developed and applied in order to systematically map the complete spectrum of lamina-protein and -chromatin interactions. Such approaches are crucial to pinpoint the biological function of the lamina and pinpoint molecular defects for specific laminopathies. In this review we provide an overview of current technologies aimed at identifying protein- and chromatin-interactions at the lamina. We focus on proteome- and genome-wide unbiased approaches, with particular emphasis on technical advantages and potential pitfalls in the context of subsequent mass spectrometry,[8,9] high-throughput microscopy and mass sequencing analysis.[10] The methods discussed, each with its strengths and weaknesses, are all complementary but they all contribute to increasing our knowledge of function of lamins and the nuclear periphery.

Protein Interactions

Visual screens. A basic approach to identifying lamina proteins is by visual inspection of the localization of candidate proteins. In

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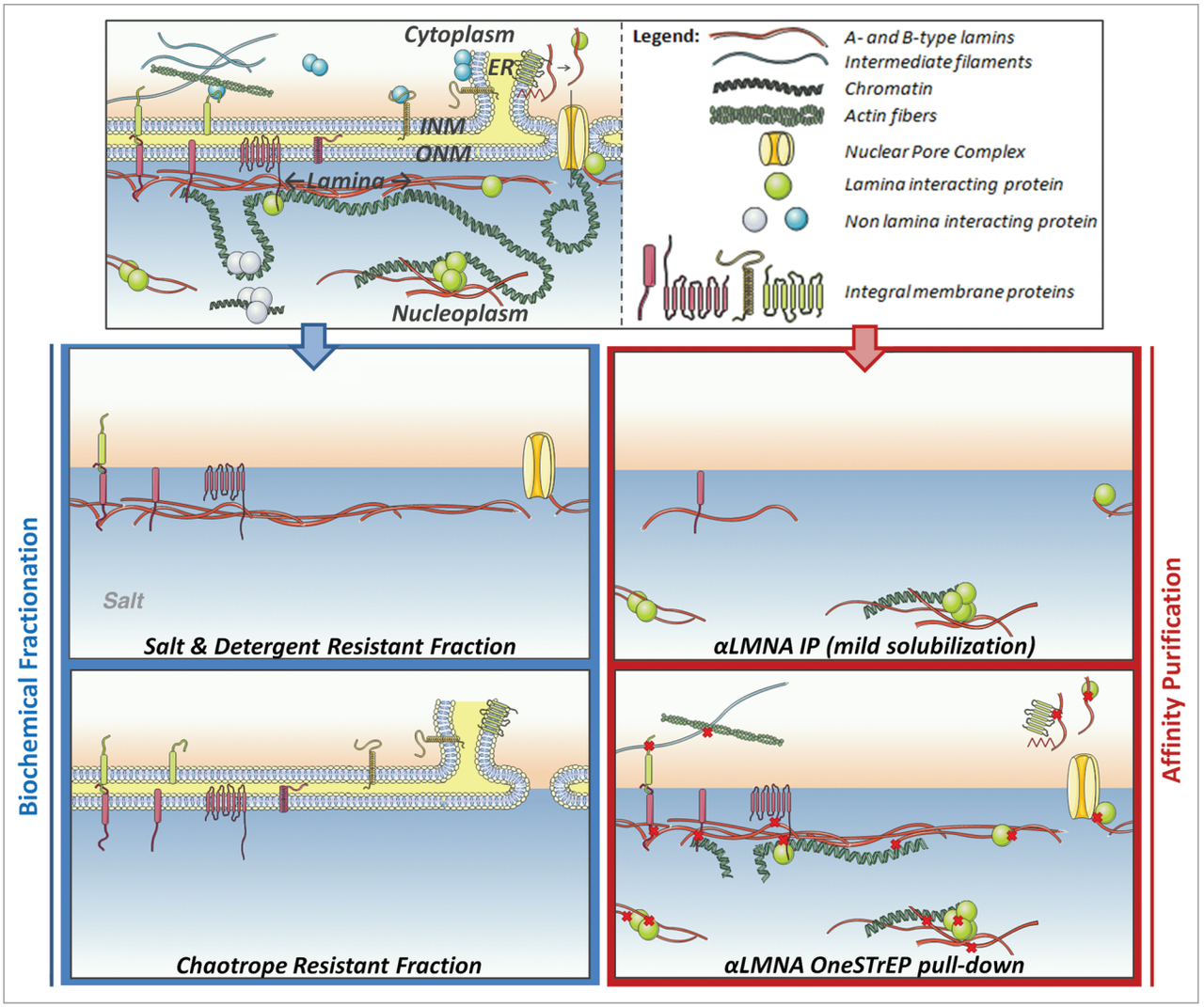

Figure 1. A schematic view of lamina and lamina-interacting protein fractions purified by various techniques. The nuclear periphery consists of an inner nuclear membrane (INM), outer nuclear membrane (ONM) and is connected to the endoplasmic reticulum (ER). (Left) Salt solubilizes weakly attached lamina proteins, but not the nuclear lamina. Detergents preferentially dissolve membrane proteins that are not anchored in the detergent resistant lamina.[28,65] Chaotropes and alkaline extraction generate an insoluble fraction mainly consisting of integral membrane proteins.[65] Immunoprecipitations (right) with an antibody directed against lamin A/C, using mild lysis conditions (for example 0.1% NP-40, 250 mM NaCl[12]), preferentially dissolve and precipitate nucleosoluble A-type lamins and protein interactors.[35] For a lamin A OST pull-down assay,[46] cross-linking, indicated by crosses, captures protein-protein and protein-chromatin interactions and allows solubilization of the total lamin A/C pool while preserving interactions.

visual screens putative lamina proteins are expressed using specific tags and their localization is then detected either using live cell imaging or indirect immunofluorescence. The use of fluorescent or other epitope tags has made it possible to systematically visualize the localization of a large number of putative lamina proteins and lamin-interacting proteins by high-throughput microscopy. The most commonly used tags are GFP and Myc. The foremost advantage of visual screens is the instant registration of dynamic or abnormal changes in subcellular protein-complex localization in the context of changed cell physiology or protein mutation, respectively (**Table 1**). Capture of such dynamic behavior has been successfully applied[11] to the study of the nuclear lamina; lamin A interactions with Rb, c-FOS and SMAD2 occur only under specified conditions, like proliferation, differentiation and TGFβ stimulation.[12,13] Sensitivity, specificity and spatial resolution in cell-based imaging are somewhat confounded by the necessity to co-stain for relevant subcellular domains as well as low cellular abundance of NE proteins.[14-17] Bioinformatic approaches can assist in visual screens by selecting for predicted membrane proteins[18] localized at the NE.[19]

A powerful and more sophisticated variation of a visual screen to identify novel lamin interaction partners is the use of GFP-fusion protein libraries. In this approach collections of GFP-tagged fusion proteins are expressed, screened by high-throughput microscopy for subcellular localization, and clones of interest are subsequently sequenced to determine the identity of the expressed protein. Rolls et al. successfully applied a heterologous promoter-driven GFP-fusion library to screen 40,000

Table 1. Techniques to identify protein interactions at the nuclear lamina

Technique category	Technique	In vitro system	In vivo system	Endogenous target	Exogenous target	Interactors bind: Direct	Indirect	Strong	Weak	False positives	Advantages	Disadvantages	References
Visual screens	*Visualize tagged proteins*	-	+	±	+	+	+	+	+	-	• No fractionation of NE compartment required • Possible to identify multiple cellular locations • Possible to screen for stimuli influencing subcellular localization	• No enrichment for NE proteins • Co-staining for organelle specific markers necessary • Weakly expressed endogenous proteins potentially not visualized • Protein overexpression can result in mislocalization • Tag can influence protein localization	11, 14-16, 20, 22, 66
Biochemical Fractionation	*Protein Correlation profiling*[*]	-	+	+	-	+	+	+	±	+	• Applicable on crude extracts, thus useful for organelles difficult to purify • Copurification provides information about protein complex composition	• No clear cut off values for different organelle fractions • Organelle specific markers necessary • Difficult to compare different samples • Influence of mutations on protein interactions difficult to determine	25
	Differential Extractions	-	+	+	-	+	+	+	-	±	• Specifically enrich for NPC, lamina or integral INM proteins • Ability to detect a large part of the complete NE proteome • No organelle specific markers necessary	• Background due to co-isolation of non-NE membranal fractions. • No information about protein interactions and complexes composition provided • Needs optimization per tissue type • Interactions dependent on chromatin, as well as weakly attached NE proteins less well detected. • Influence of mutations on protein interactions difficult to determine	27-28, 67-69

Legend: No (-), endogenously expressed, but genetically modified (±), Yes (+). For 'false positives'-column: low (-), moderate (±), high (+); AB, Antibody.
[*]Data not analyzed for subnuclear structures yet.

clones and test in an unbiased fashion whether they localized to the nuclear periphery.[20] In doing so they discovered the NE membrane protein Nurim, a six-transmembrane spanning INM protein with potential isoprenylcysteine carboxymethyltransferase enzymatic activity for Caax-motifs.[20,21] A drawback of protein overexpression is possible mis-localization of the fusion protein; there is an obvious need for analysis of the effect of protein expression levels on its localization and the validation of each hit by analysis of the endogenous protein.[15,22] A key advantage of visual screens is that interactors which may associate with the small, but significant pool of lamin components present in the nucleoplasm, can be distinguished from those that interact with the peripheral pool of lamins. In a more physiologically relevant approach, Bickmore and colleagues identified the NE associated protein Lyric/AEG-1, an apoptosis and cell growth implicated transcriptional regulator, by using gene trapping to insert 1,350 reporters into active genes.[11,23] In this approach, endogenously expressed genes were spliced onto a genomically integrated LacZ cassette, which allowed visualization of the resulting fusion proteins by X-gal staining and β-galactosidase immunohistochemistry.[11] Although such visual screens are becoming increasingly feasible, they are relatively labor intensive and slow.

Biochemical fractionation. NE proteins are highly lipophylic and lamina proteins are strongly resistant to high concentrations

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Technique category	Technique	In vitro system	In vivo system	Endogenous target	Exogenous target	Interactors bind: Direct	Indirect	Strong	Weak	False positives	Advantages	Disadvantages	References
Affinity Purification	*Classic Immuno-precipitation*	-	+	+	+	+	+	+	-	±	• Specifically enrich for interactors of protein of interest • Possibility to gradually elute interactors, which provides information on protein complex composition • Influence of mutations on protein interactions can be determined • IP possible for specific post-translational modifications	• Difficult to fully solubilize intact protein complexes • No direct information provided on (multiple) localization(s) protein of interest • Quality highly dependent on AB	5, 12-13, 33, 36, 38, 40-45, 61, 70-73
	Immuno-precipitation with bait	-	+	-	+	+	+	+	-	±	• Specifically enrich for interactors of protein of interest • Possibility to gradually elute interactors, which provides information on protein complex composition • Efficient and specific precipitation, because bait is translated in vitro • Influence of mutations on protein interactions can be determined	• Difficult to fully solubilize intact protein complexes • No direct information provided on (multiple) localization(s) protein of interest • Quality highly dependent on AB • IP not possible for specific posttranslational modifications • Protein interactions are possible not native	33, 40, 44-45, 71
	OneSTrEP Pull-down	-	+	-	+	+	+	+	+	±	• Specifically enrich for interactors of protein of interest • Protein complexes can be fully solubilized intact • AB-free high affinity purification • Influence of mutations on protein interactions can be easily determined • Interactions dependent on chromatin, as well as weakly attached NE proteins detected.	• No direct information provided on (multiple) localization(s) protein of interest • Gradual elution of interactors not possible due to cross-linking. Therefore no extra information on protein complex composition • Pull-down not possible for specific posttranslational modifications	6, 46
Others	*Yeast Two-Hybrid*	-	+	-	+	+	-	+	+	+	• Interactions can be easily mapped to protein domains • Influence of mutations on interactions can be easily determined • Tissue specific libraries can be used to identify tissue specific interactors	• Found interactions do not necessarily occur in vivo • No information provided on protein complex composition or subcellular localization • Inability to study posttranslational modifications	6, 13, 37, 45, 5, 71, 7

Legend: No (-), endogenously expressed, but genetically modified (±), Yes (+). For 'false positives'-column: low (-), moderate (±), high (+); AB, Antibody.
*Data not analyzed for subnuclear structures yet.

of salts and detergents. These biochemical characteristics are exploited in fractionation studies to separate NE and lamina proteins from other subnuclear domains. Such isolation drastically increases the frequency of detecting NE and lamina proteins by unbiased biochemical methods e.g., mass spectrometry (MS) (**Table 1**). Combined with recent MS advances in complex protein mixture analysis,[9,24] such fractionation studies have the potential to contribute significantly to the identification of the full lamina proteome. As MS analysis itself cannot distinguish lamina proteins from contaminants, fraction purity is crucial.

Various assays have been developed to fractionate NE and lamina proteome subsets, each with a specific trade-off between purity and the amount of background proteins.

Protein Correlation Profiling (PCP) was developed to determine subcellular protein localization in crude extracts, separated by rate-zonal centrifugation into fractions which are subsequently analyzed by MS analysis (**Table 1**).[25] In essence, the technology relies on co-detection of proteins known to reside in the organelle of interest and novel proteins.[25] The main advantage of PCP is its ability to detect multiple co-segregating proteins in a complex mixture without the need to fully isolate and highly purify subcellular fractions. However, PCP comes at a price: as many subcellular domains are only partially separated by centrifugation, non-specific interactors co-purify and separation of true interactors relies strongly on computational analysis. Follow-up studies to characterize the properties of identified proteins are imperative. Although PCP analysis has not been applied yet to distinguish and define nuclear lamina/envelope protein domains, the fact that differences in rate-zonal resolving properties have previously been used to purify NE fractions,[26] makes PCP a promising technique to identify NE and lamina proteins.

In comparison to PCP, differential extraction assays use more highly purified fractions for MS analysis and consequently reduce non-specific co-purification (**Table 1**). In general, biochemical fractionation of the NE starts with isolation of nuclei, removal of non-NE membrane fractions by centrifugation and digestion of chromatin to remove nucleoplasmic contents. Crude NE and lamina fractions are subsequently extracted in salt, detergent and chaotrope or alkaline buffers to further remove different types of proteins. Salt preferentially dissolves chromatin-bound proteins and other non-membrane, weakly attached lamina proteins, but not lamina proteins (**Fig. 1**). Detergents (Octyl glucoside, Trx-100, Empigen BB) mimic the lipid bilayer environment and particularly dissolve membrane-associated proteins, except those that are anchored to the detergent resistant lamina (**Fig. 1**). In this manner Cronshaw et al. successfully identified 6 novel NPC components, among which ALADIN, the gene mutated in the triple-A or Allgrove syndrome.[27] Chaotropes (urea, thiourea) and alkalines (NaOH) are used to solubilize cytoskeletal, chromatin and lamina components, while leaving integral transmembrane proteins embedded in the insoluble membrane fraction (**Fig. 1**). As such lamina and lamina-anchored INM/NE proteins are extracted by combined application of salt and detergent (**Fig. 1**; reviewed in ref. 29 and 30), whereas integral INM-, ONM- and ER-membrane proteins are purified by chaotrope or alkaline extraction (**Fig. 1**).

Despite increased sample purity by applying differential extractions, these fractionation studies are still hampered by co-purification of non-specific interactors, in particular from the peripheral ER, which is continuous with the ONM and therefore difficult to separate from the NE and lamina. To further reduce false positive hits, Dreger et al. compared salt, detergent and chaotrope/alkaline extractions, and were able to eliminate ER contaminants in chaotrope/alkaline resistant fractions.[28] This strategy identified 19 previously unknown and putative integral INM proteins including Unc84a (Sun1), LUMA and two LAP2 isoforms.[28] A disadvantage of this comparative approach is that selection is based on the assumption that all INM and lamina proteins have similar biochemical extraction characteristics. However, several lamina anchored INM proteins, like emerin, LBR and LAP1, behave biochemically very different in detergent or chaotrope-based extraction.[28]

In an extenstion of purification methods, subtractive approaches can be used to filter out ER residing proteins. In these methods proteins identified in non-NE/lamina fractions, thus enriched for background, are subtracted from proteins detected in differential NE/lamina extracts. Schirmer and colleagues used microsomal membrane fractions as a source of background proteins, as these ER-rich fractions are easily obtained and can be prepared free of nuclear membranes.[29] By combining differential fractionation and subtractive proteomics, they identified 67 previously unidentified NE transmembrane proteins. The disadvantage of using a reference background source is that proteins that reside in both the ER and NE, like AEG-1/Lyric, Sec13 and Torsin A, are inadvertently discarded.[30-32] This is a serious concern, since it is now estimated that one third of cellular proteins have multiple organellar localizations.[25]

Affinity purification. An alternative approach to identifying lamina interactions is affinity purification. In these approaches a protein of interest is epitope tagged and the bait protein is affinity purified using antibodies against the tag (**Table 1**). Affinity purification can identify and distinguish bound protein complexes from each other by co-elution and MS analysis. Such gradual elution was used for example to separate emerin-interacting RNA processing, signaling and chromatin remodeling complexes.[33] In addition, it is possible to study interactions in the context of post-translational modifications by using specific antibodies directed against them, for example phosphorylation-dependent LBR-p32/p34,[34] lamin A-Rb and -Smad2,[12] interactions. In contrast to biochemical fractionation, in which fractions are generated under denaturing conditions, the main challenge in affinity purification assays is to extract as many NE and lamina proteins while leaving protein interactions intact. Overly stringent solubilization dissolves many proteins at the cost of disrupting complexes, while excessively mild conditions do not dissolve all relevant interactors. Various strategies have been applied to find a good balance for this trade-off.

In classic immunoprecipitations (IPs) solubilization conditions are optimized for the protein of interest (**Table 1**). Low amounts of detergents and salt preferentially solubilize nucleoplasmic pools of proteins (**Fig. 1**), as described to exist for lamin A,[35] and were mainly applied to study easy extractable, weakly bound NE interactors (Smad2, PP2A, Rb, Ubc9, hnRNP1, EGF1, SREBP1; See **Table 2**).[5,12,36-38] Increasing amounts of detergents, salts and the solvent glycerol[39] successfully solubilize protein complexes of well-anchored NE and lamina components (LAP2β, Emerin, Nesprin2, Lamin B; **Table 2**), although sometimes at the cost of disrupting interactions (LaminA/B1/B2-LAP2β)[40] (**Table 2**). Highly stringent conditions were applied when studying NPC proteins as they were assumed to be highly stable structures (**Table 2**).[41-43] Even though NPCs apparently better withstand stringent extraction, increased stringency of washing buffers

Table 2. Solubilization conditions to identify protein interactions at the nuclear lamina.

Affinity Purification Category	Interactions	Cross-linked (Y/N)	Sonicated (y/N)	Lysis buffer: Detergents: SDS (%)	NP-40 (%)	Trx-100 (%)	Salts: Sodium Deoxych.(%)	NaCl (mM)	KCl (mM)	Others: Glycerol (%)	DTT (mM)	DNAse (Y/N)	RNAse (Y/N)	Stringency washing buffer	Footnote	References
Classic IP - Native complex	Man1/SMAD2	N	Y		0.1						1	N	N	Similar	1	(36)
	LMNA&C/ SMAD2/ PP2A/Rb	N	Y		0.1			250				N	N	Similar	2	(12)
	LMNA&C/Progerin/ Ubc9/ hnRNPE1/EGF1	N	N			1.0						N	N	Similar		(37)
	LMNA/Sun1	N	N			1.0		50				N	N	Similar		(71)
	preLMNA/ SREBP1	N	N		1.0			100				N	N	Similar	3	(38)
	LMNA/SREBP1	N	N			1.0		150				N	N	Similar		(5)
	LAP2β/LMNA/ LMNB1/LMNB2	N	N			1.0		150				N	N	150-300 mM NaCl	4	(40)
	LMNA/cFOS	N	N		1.0			150		10				500 mM NaCl		(13)
	Emerin/Nesprin2	N	N			1.0	0.50	150				N	N	Similar	5	(45)
	Nup153/p250/p62	N	N			0.5		500			1	N	N	0-0.1% SDS	6	(41)
	Nup50/MC2R	N	N		0.1*			420	1500*	25	0.5	N	N	Similar	7	(43)
	Nup96, 107, 120, 133	N	N			2.0			150		1	Y	Y	Similar	8	(42)
Classic IP -Assembled complex	BAF/Various Interactors	N	N			0.30→ 0.15		300→ 150			2	Y	N	Similar		(44)
	E(Y)2/SAGA	N	N		0.3→ 0.1			420→ 140		10	1.0→ 0.3	Y	Y	500 mM KCl		(61)
	Emerin/ Various Interactors	N	N			1.0→ 0.1		1000→ 100				N	N	Similar	9	(33)
	LAP1/LMNB	N	N			2.0→ 1.0		300→ 150			5.0→ 2.5	N	N	Similar	10	(70)

disrupts interactions (**Table 2**).[41] An additional disadvantage to be accounted for is that lysis buffers also can affect the antibody/epitope-interaction. Various groups therefore prefer to dilute buffer compositions after initial lysis, which combines increased solubilization with the ability of protein complexes to reassemble and antibodies to bind under sequentially milder conditions. This strategy was used to identify unknown interactors for BAF and Emerin (**Table 2**).[33,44] Other limitations of antibody-based methods include the unavailability of IP-able antibodies,[36] antibodies that recognize multiple epitopes (MAN1 antiserum),[36] antibodies that cross-react undesirably with non-mature forms of a protein (prelamin A versus lamin A)[38] or even disrupt protein interactions (laminA/B2-LAP2β).[40]

To avoid the use of antibodies, precipitations can be performed using bacterially expressed and purified baits, conjugated to beads prior to incubation with solubilized protein extracts. Fusing the bait to an epitope tag contributes to high quality purification of the bait and efficient precipitation of interactors from protein extracts (**Table 1**). This approach was combined with mild lysis, for BAF and emerin interactors,[33,44] or more stringent buffers, for lamin-LAP2β and -nesprin 2 interactions (**Table 2**).[40,45] In accordance with mild solubilization, identified BAF and emerin interactors represented many proteins that also

Table 2. Solubilization conditions to identify laminar protein interactors (continued)

Affinity Purification Category	Interactions	Cross-linked (Y/N)	Sonicated (y/N)	Lysis buffer: Detergents: SDS (%)	NP-40 (%)	Trx-100 (%)	Salts: Sodium Deoxych.(%)	NaCl (mM)	KCl (mM)	Others: Glycerol (%)	DTT (mM)	DNAse (Y/N)	RNAse (Y/N)	Stringency washing buffer	Footnote	References
IP with bait	BAF^{Vitro}/ Various Interactors	N	N		0.5*	0.1		150			2	Y	N	Similar	11	(44)
	Emerin^{Vitro}/ Various Interactors	N	N			0.2			20	25	0.5	N	N	250mM NaCl;0.1 %Trx-	12	(33)
	LMNC^{Vitro}/ MOK2	N	N		0.1			420→ 150		25	0.5	N	N	Similar	13	(71)
	XLAP2β^{Vitro}/ LMNA/ LMNB1/ LMNB2	N	N			1.0		150				N	N	Similar	14	(40)
	Nesprin2^{Vitro}/ LMNA/LMNC	N	N			1.0		150				N	N	Similar		(45)
OST Pull-down	LMNA/RBBP4	Y	Y	1.0→ 0.1		1.0		150				N	N	2M NaCl; 2%Trx-100		(6)
	LMNA/ Progerin/ Various Interactors	Y	Y	1.0→ 0.2		1.0		150				N	N	2M NaCl; 2%Trx-100	15	(46)

Legend: Identified interactions are sorted by type of affinity purification used [Classic Immunoprecipitation (IP) on native protein complexes, Classic IP on re-assembled complexes, IP using a bait, OST pull-down] and stringency of used lysis buffer. Footnotes:

1. Antiserum recognizes MAN1, LAP2β, unidentified epitopes.
2. LMNA AB preferentially precipitates LMNA compared to LMNC.
3. LMNA AB also (reduced) affinity for preLMNA.
4. Washes up to 0.3M NaCl resulted in complex dissociation; Protein domains were expressed and used for IP as well; LAP2β for 90% solubilized, LMNA, LMNB1,2 for ±50%;Certain ABs disrupt Lamin-LAP2β interaction.
5. Very small part of solubilized Nesprin2 was precipitated.
6. 0.1% SDS wash fully disrupts p250 and p62 interaction and leaves approximately a 1/3rd of the Nup153 interaction intact.
7. *Cells pre-extracted in 0.1% NP40 & 1.5M KCl. Insoluble pellet lysed in described lysis buffer (without NP40 and KCl).
8. Increasing NaCl to 0.5M did not drastically improve NPC solubilization; Increasing Trx-100 from 1% to 2% increased NPC solubility from ±50 to 80%.
9. Novel interactors identified by MS.
10. 10% sucrose included as well in lysis buffer; Lysing directly in lysis buffer with 1% Trx-100,150 mM NaCl resulted in worse solubilization of membrane proteins.
11. Cells were pre-extracted by 0.5% NP40. Novel interactors identified by MS.
12. Novel interactors identified by MS ; Only residues 1-122 of emerin were translated.
13. Protein complex re-assembled due to dialysis of lysis buffer. Listed within this category because LMNC was translated in vitro.
14. Protein domains were in vitro translated and used for IP as well.
15. Novel interactors identified by MS.

reside outside the nuclear periphery (PARP, HP1gamma, RBBP4,7).[40,45] Another advantage of using a bait is the ability to pinpoint interactions to relevant protein domains, as the bait does not have to be incorporated in vivo and therefore cannot mislocalize, as described to occur for LAP2β constructs.[40] The main disadvantages of IPs is that solubilization issues still remain and interactions formed in vitro do not necessarily occur in vivo.

A major step forward in overcoming solubilization problems is the OneSTrEP (OST) pull-down assay,[6,46] which combines the use of a biotin resembling OST-tag[47,48] with mild cross-linking of cells prior to solubilization (**Table 1**). Cross-linking allows extraction of the total lamin A/C pool[46] (**Fig. 1**) while leaving protein interactions intact. The OST-tagged protein and its interactors can then be highly efficiently precipitated under denaturing conditions using a high-affinity, engineered streptavidin analogue (**Table 2**). Stringent washes reduce background, especially relevant for A-type lamins, known to be "sticky" proteins and reported to precipitate in negative pull-down controls as well.[44] The main advantages of the OST pull-down are that it can be used to identify a full in vivo interactome of a protein regardless of its subnuclear position, detect the effect mutations have on these interactions and identify weak interactors.[46] OST pull-downs have been used to compare protein interactions

for lamin A and progerin, an HGPS causing lamin A mutant and detected a decreased interaction for progerin with NPC components.[44] The main disadvantages of this approach are that it is not possible to study endogenous proteins and that cross-linking does not allow gradual elution and thereby separation of interacting protein complexes (**Table 1**).

Yeast two-hybrid. An alternative to affinity purifications is yeast two-hybrid (Y2H) (**Table 1**), in which a direct interaction between a DNA-binding domain fused bait and a co-expressed transcriptional activating domain fused prey allows growth under restrictive conditions. When the primary interest is to identify weak and direct interactors, Y2H is useful as bait and prey are expressed by strong exogenous promoters, protein solubilization is not required and weak interactions are sufficient to allow restrictive growth. The focus on direct protein interactions, which might best reflect the core activities of the protein of interest, restricts the mapping of a complete interactome. Protein fragments can easily be used as bait since they don't have to be incorporated in vivo, and have been applied to describe interactions between the specific domains of the nuclear envelope proteins otefin, lamin A, nesprin.[45,49,50] The benefit of choosing the exact bait composition can further be exploited by choosing domains involved in disease mechanisms, like the 50 amino acid deleted region in progerin, shown in a Y2H screen to interact with the NURD chromatin remodeling complex component Rbbp4.[6] Disadvantages of Y2H assays are the lack of information on protein complex composition, the inability to study posttranslational modifications and the large amount of false positives identified. The large amount of background can be caused by endogenous transcriptional activity of bait or prey proteins as reported for cFOS domains used to map lamin A interaction,[13] bait or prey proteins affecting yeast growth under restrictive conditions, and the fact that investigated interactions may never occur in vivo (**Table 1**). In addition, Y2H approaches for lamin proteins are particularly difficult since expression of lamin-fusion proteins in *S. Cerevisiae* has detrimental effects on the organism.

Chromatin-Interactions

In addition to protein-protein interactions, the importance of interactions between chromatin and the lamina is increasingly appreciated. In particular, many lamin proteins are now known to directly or indirectly interact with chromatin and chromatin defects are a hallmark of several laminopathies.[51-53] These observations have catalyzed the development of unbiased screening techniques for chromatin interactions at the NE. A broad distinction can be made between assays using affinity purification and those based on enzymatic activity (**Fig. 2 and Table 3**).

Affinity based approaches: ChIP & OST pull-down. Chromatin-protein interactions are most commonly interrogated using chromatin-immunoprecipitation (ChIP) methods. In this approach, a protein of interest is cross-linked to chromatin and immunoprecipitated using a specific antibody against the protein. The DNA is then identified either by targeted PCR methods or by genome-wide microarray or sequencing approaches. The major difference between conventional IPs and ChIP is the addition of a cross-linking step prior to solubilization of intact protein-chromatin complexes. Cross-linking provides the advantage of combining ultra-sonication and stringent lysis, to shear DNA and dissolve NE proteins (**Fig. 2**), with good preservation of protein-chromatin interactions (**Table 3**). Just as for classic IPs, lysis buffers still need to be adjusted to the strength of the epitope-antigen interaction. For this reason, initial ChIP studies were performed on Myc-tagged NPC proteins in *S. Cerevisae*,[54] as NPCs are easily dissolved in the absence of nuclear lamina and high quality ChIP-suited Myc antibodies are commercially available. For the INM protein Src1, a MAN1 resembling protein, interactions with (sub)telomeric regions were identified in yeast using a high affinity protA-system.[55,56] Silver et al. used endogenous Nup93 in HeLa cells by dialyzing the initial lysis buffer to a milder variant prior to incubation with antibodies.[57] The foremost advantage of using antibodies is the ability to study endogenous proteins and chromatin interactions in the context of posttranslational modifications (**Table 3**).

A modification of the classical ChIP approach is the use of the OneSTrEP tag (OST) pull-down which enables high affinity precipitation of OST-tagged proteins under denaturing conditions completely dissolving A-type lamins, comparable to the use of OST tags used for pull-down of proteins (**Table 3**).[46]The OST pull-down for identification of chromatin interactions is highly similar to that for detecting protein interactions and only includes slight changes in sonication and washing conditions.[46] Although OST pull-downs have the advantage of easy solubilization and high affinity pull-down without the use of antibodies, which in the case of lamin A have not been ideal in ChIP experiments, a limitation is the inability to directly study endogenous proteins and posttranslational modifications (**Table 3**).

Enzymatic activity based approaches: DamID, in vivo ChEC, ChIC. DamID is an enzyme-based method for the in vivo mapping of chromatin-protein interactions. In DamID a protein of interest is fused to a DNA adenine methyltransferase (Dam) and expressed. Upon binding of the fusion protein to chromatin, the Dam activity marks in the vicinity bound chromatin by methylation, thereby enabling selective DpnI restriction in vitro. The marked sites can then be identified by targeted PCR or, more commonly, by genome-wide microarray analysis and deep-sequencing[58] (**Fig. 2**). The main advantage of using a tag that enzymatically marks DNA is that only isolation of DNA, not of intact protein/chromatin complexes, is required, thus eliminating any issues related to interaction stability. In addition, there is no need for cross-linking, thereby avoiding potential fixation artifacts. These characteristics made DamID the first technique to characterize and compare chromatin interactions for the relative insoluble lamin B and emerin proteins in a genome-wide fashion and resulted in the characterization of lamin associated domains (LADs) which define regions of the genome that preferentially interact with the lamina.[58,59] Disadvantages of DamID include the inability to study posttranslational modifications, a slightly reduced resolution compared to alternative assays and potential interference of the tag with protein localization or function (**Table 3**).[60] In addition, since DamID relies on the expression

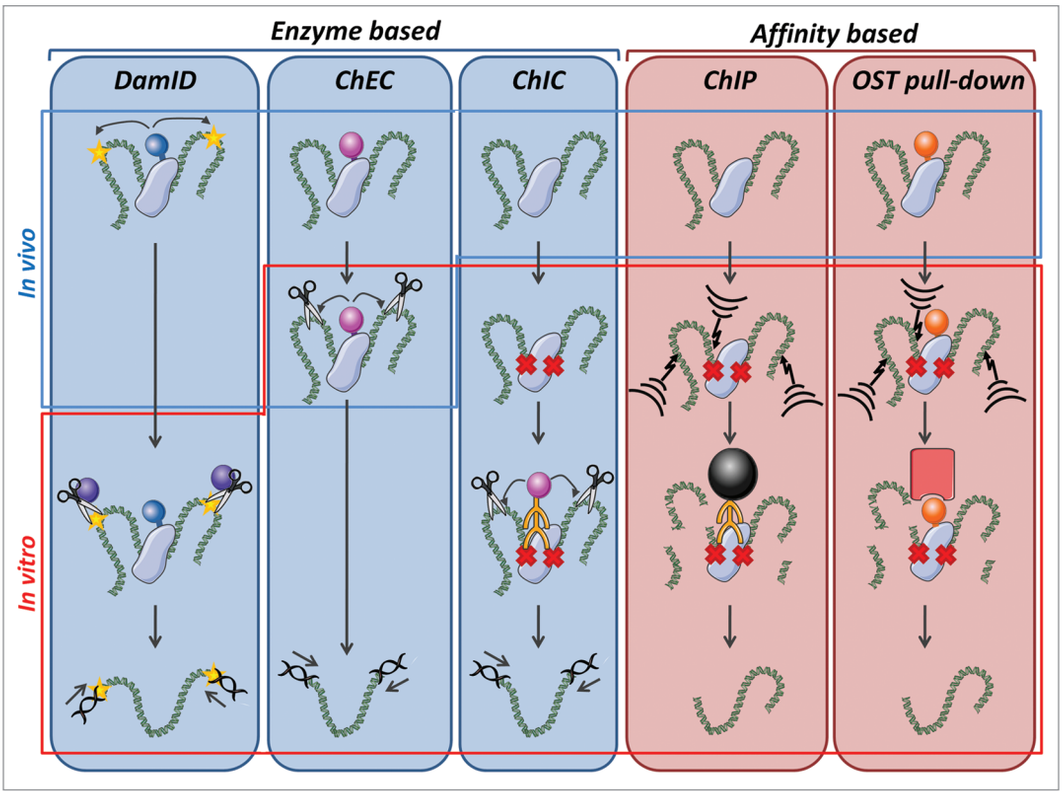

Figure 2. Schematic overview of techniques to identify chromatin interactions, which are categorized in enzymatic- and affinity-based approaches. For DamID[58] a DNA adenine methyltransferase (Dam) tag (ball on stick) is fused to the protein of interest and adenylates (star) bound chromatin in vivo, enabling in vitro selective DpnI (scissor) restriction and subsequent amplification of restricted chromatin by ligation mediated PCR (LMPCR). For in vivo chromatin endogenous cleavage (ChEC)[63] a protein of interest is fused to a micrococcal nuclease (MNase) tag, which introduces DNA double strand breaks (scissors) upon introduction of calcium chloride to weakly permeabilized cells. Due to the mild permeabilization of cells prior to addition of calcium chloride for activation, the MNase digestion step is indicated as being partially in vitro and in vivo. Restricted DNA is amplified by LMPCR. For chromatin immunocleavage (ChIC)[63] cells are cross-linked (crosses). In vitro, MNase-conjugated antibody interacts with the epitope of interest and induces DNA breaks enabling LMPCR amplification of cleaved chromatin. For chromatin immunoprecipitation (ChIP) chromatin-protein interactions are cross-linked and chromatin is randomly sheared, typically by ultrasonication, (lightning arrow and stripes). Antibodies are used to precipitate the endogenous protein of interest with the help of antibody binding beads (big ball). In a OneSTrEP (OST) pull-down a OST-tagged protein is expressed.[58] Cells are cross-linked and ultrasonicated. The OST-protein is highly efficiently precipitated by a streptactin matrix (big square).

of an enzymatically active fusion protein, it monitors chromatin interactions over a relative long period of time (app. 24 hours) and therefore is less useful to detect rapid interaction changes and dynamic reorganization of chromatin. Due to the DNA binding activities and high enzymatic activity of Dam, tagged proteins can only be expressed in trace amounts in order to prevent saturation of non-targeted DNA methylation.[61] This makes it not possible to study chromatin interactions in a dosage dependent manner, which could be relevant for diseases in which phenotypes are dependent on the amount of protein, such as HGPS.[62]

An alternative method to measure protein-chromatin interactions is ChEC (chromatin endogenous cleavage). In this approach, micrococcal nuclease (MNase) is fused to a protein of interest and expressed. The fusion protein is recruited to its endogenous sites on chromatin where the MNase introduces double strand breaks at nuclease hypersensitive sites (HS).[63,64] The MNase tag remains inactive under physiological Ca^{2+} concentrations, which provides the ability to selectively turn its activity on in vivo by addition of calcium chloride to mildly permeabilized cells. Cleaved chromatin can either be directly used to map HS sites by indirect end-labeling and Southern blotting, or is first selectively amplified by ligation-mediated PCR prior to genome-wide microarray analysis and deep-sequencing (**Fig. 2**).[63] Laemmli and colleagues used this approach to map chromatin interactions of the nuclear pore complex protein Nup2 and found that Nup2-gene promoter interactions typically are an early event of gene activation and are independent of transcription.[63] Control over MNase activity and relative short times needed for digestion make this assay suitable for detection of rapid changes in interactions. The major strength of in vivo ChEC is that there is no need to dissolve intact protein/chromatin complexes and information on chromatin structure is obtained by mapping HS (**Table 3**). In comparison to DamID higher expression levels of MNase-tagged proteins can be used, although at very high expression levels background issues were reported.[64] ChEC can also be modified to study posttranslational modifications as the MNase tag can also be conjugated to an antibody of interest. This in vitro method is

Table 3. Techniques to identify chromatin interactions at the nuclear lamina

Technique category	Technique	System		Studied proteins		Captures		Advantages	Disadvantages	Extra information	Refs.
		In vivo	Genome wide	Endogenous	Overexpressed	Snapshot	Time period				
Enzyme Activity	*DamID*	+	+	-	+	-	+	• No need for solubilization of protein/DNA complexes • No formaldehyde fixation required	• Relative low resolution • Target protein needs to be expressed at very low levels • Not possible to study posttranslational modifications	• Tag is active under physiological conditions • Resolution of ~1000 bp • Dam-tag ~32 kDa	58,60
	In vivo *ChEC (Chromatin Endogenous Cleavage)*	+	+	-	+	±	±	• No need for solubilization of protein/DNA complexes • No formaldehyde fixation required • Southern blot analysis provides extra information about hypersensitive sights	• Dependent on the presence of DNA restriction sites • Target protein cannot be expressed in high levels • Not possible to study posttranslational modifications	• Tag is inactive under physiological conditions • Resolution of ~100 bp • MN-tag ~18 kDa	63-64
	ChIC (Chromatin Immuno-cleavage)	+	+	+	+	+	-	• No need for solubilization of protein/DNA complexes • Southern blot analysis provides extra information about hypersensitive sights • Possible to study rapid changes of interaction • Endogenous enzymatic activity impossible • Antibody allows to study posttranslational modifications	• Dependent on the presence of DNA restriction sites • Interacting DNA that cannot be reached by tag enzymatic activity remains undetected • Antibodies may need separate optimization • Formaldehyde fixation required	• Tag is inactive under physiological conditions • Resolution of ~100 bp	63-64
Affinity Based	*Chromatin Immuno-precipitation*	+	+	+	+	+	-	• Antibody allows to study posttranslational modifications • Possible to study rapid changes of interaction	• Dependent on antibody quality • Formaldehyde fixation required • Solubilization of protein/DNA complexes required	• Resolution of ~500 bp	54, 57, 75-78
	OneSTrEP Pull-down	+	+	-	+	+	-	• High affinity precipitation without need for a Antibody • Near complete solubilization of protein/DNA complexes possible	• Formaldehyde fixation required • Not possible to study posttranslational modifications	• Resolution of ~500 bp • OST-tag ~3 kDa	

Legend: No (-), Moderate time period (±), Yes (+).

referred to as chromatin immunocleavage (ChIC). In ChIC cross-linked cells are lysed and incubated with MNase-coupled antibodies that bind to the epitope of interest after induction of DNA cleavage by Ca^{2+} (**Fig. 2**). ChIC is a hybrid between affinity- and enzyme-based approaches in that it uses cross-linking and antibodies, but does not need to fully dissolve and precipitate intact protein-chromatin complexes due to the use of enzymatic activity, which specifically marks bound DNA (**Table 3**).

Concluding Remarks

The characterization of structural and other functional components in the nuclear lamina is vital for our understanding of

higher-order chromatin organization, transcription, DNA replication and various other nuclear processes. Recent development of powerful techniques to map protein and chromatin interactions has begun to reveal these roles.

Several approaches to identify the interaction network at the nuclear periphery are now available. These methods are all complementary and each has its own usefulness and limitations. Ideally, one would map the interactions of proteins and chromatin using multiple, complementary techniques. At present this is practically often not feasible, however, as interaction-detection methods are improved, it should become possible to interrogate interactions by multiple means. For now, the choice of method often relies on the particular question to be addressed. When it is important to identify multiple sub-cellular localizations of a protein, visual screens are the best option. Biochemical fractionation studies best assist in revealing a full proteome. A more detailed impression of an individual protein's interactome can be obtained by classic IPs to study endogenous proteins, by OST pull-down to identify weak and relative insoluble proteins and by IPs using a bait to distinguish individual protein complexes. Mapping interactions of direct and weak interactors to protein domains can best be done by Y2H. For DNA interactions a careful choice has to be made between the need for studying endogenous proteins and posttranslational modifications (ChIP, ChIC), obtaining extra information on chromatin structure (In vivo ChEC, ChIC), full protein solubilization and obtaining an instant snapshot of interactions (OST pull-down), or not dissolving protein/chromatin complexes and capturing interactions over a longer period of time (DamID).

Even though the overlap between various chromatin techniques is slightly bigger than for protein techniques, in both fields a combinatorial or comparative use of techniques, as well as the target proteins they are applied on, will lead to more reliable results and provide a better understanding of the NE. These methods are becoming increasingly routinely used in many laboratories and there is no doubt that proteome and genome-wide mapping method and screening for mutation-induced interaction changes will play a key role in unraveling nuclear lamina function and laminopathy disease mechanism.

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