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The extended pluripotency protein interactome and its links to reprogramming

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Abstract

A pluripotent state of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) is maintained through the combinatorial activity of core transcriptional factors (TFs) such as Oct4, Sox2, and Nanog in conjunction with many other factors including epigenetic regulators. Proteins rarely act alone, and knowledge of protein-protein interaction network (interactome) provides an extraordinary resource about how pluripotency TFs collaborate and crosstalk with epigenetic regulators in ESCs. Recent advances in affinity purification coupled with mass spectrometry (AP-MS) allow for efficient, high-throughput identification of hundreds of interacting protein partners, which can be used to map the pluripotency landscape. Here we review recent publications employing AP-MS to investigate protein interaction networks in ESCs, discuss how protein-protein connections reveal novel pluripotency regulatory circuits and new factors for efficient reprogramming of somatic cells.

Introduction

Pluripotency, the capacity to differentiate into all cell types, is a defining property of embryonic stem cells (ESCs). The undifferentiated state of ESCs is maintained by a set of pluripotency factors [1]. Forced expression of these factors (i.e., the Yamanaka factors [2] Oct4, Sox2, Klf4, and c-Myc, OSKM) can reprogram lineage-committed cells back to an ESC-like state (called induced pluripotent stem cells, iPSCs), providing extraordinary potential for regenerative medicine [3,4]. Transcriptional cooperation and their regulatory networks among the pluripotency factors such as Oct4, Sox2, and Nanog have been

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extensively studied in ESCs [5,6]. Given that the proteins rarely act alone, the physical protein-protein interaction (PPI) networks of pluripotency factors should provide valuable information about how the pluripotent state is established and maintained. Here we review the recent advances in pluripotency interactome studies on understanding the intricate protein interaction networks and protein complexes surrounding several critical pluripotency factors. Three aspects are discussed in detail: the technology behind protein mass spectrometry to investigate PPIs, the emerging insights on the extended ESC protein interactome, and how the pluripotency interactome reveals novel factors for efficient somatic cell reprogramming.

Methods to study protein-protein interactions

A number of methods have been developed to examine the binary PPIs in ESCs (reviewed in ref [7]). Among those approaches, affinity purification coupled with mass spectrometry (AP-MS) has become the method of choice [8]. The conceptual view of an interactome study using AP-MS is described in Figure 1. Five years ago, label-free approaches were used to compare the abundance of purified proteins by counting the number of detected peptides. Recently, due to the advent of high-accuracy MS, stable isotope labeling approaches (such as stable isotope labeling by amino acids in cell culture, SILAC) are being employed, yielding increasing robustness and information content of quantitative proteomics data [8]. Biological replicates with forward and reverse (swapped labeling) SILAC experiments are usually needed to further enhance confidence of protein interactions [9]. Conventional protein co-immunoprecipitation assays are also necessary to validate the interacting candidates.

Pluripotency protein interactome in ESCs

The published pluripotency interactome centered on TFs and epigenetic regulators in ESCs is summarized in Table 1. In 2006, the first comprehensive interactome was conducted on a number of pluripotency proteins with a particular focus on Nanog in mouse ESCs [10]. Unlike the other pluripotency factors such as Oct4 and Sox2, which are uniform in all undifferentiated ESCs, Nanog expression is relatively heterogeneous [11]. Depletion of Nanog immediately decreases self-renewal efficiency of ESCs and leads to cellular differentiation [12]. With a high-affinity biotin/streptavidin (Bio/SA) purification combined with a high-salt eluting method, Wang *et al* identified 17 Nanog interactors with high confidence. The interaction network was expanded for the identified Nanog interactors such as Dax1 (Nr0b1), Nac1, Zfp281, Oct4, and Rex1 with the same AP-MS strategy [10]. The Nanog interactome was further extended in later studies by using biotin-, Flag tag-, and endogenous Nanog antibody-based affinity approaches [13–15]. With an improved tagging method and less stringent purification, Gagliardi *et al* [14] identified 130 Nanog interactors and dramatically expanded our knowledge of the Nanog interactome in ESCs. Many of the proteins identified are components of protein complexes involved in different machineries, especially in epigenetic regulation and chromatin remodeling. Interestingly, protein complexes with opposing functions are observed to interact with Nanog, such as histone acetyltransferase complexes (Tip60-p400, also called NuA4-HAT) and deacetylase complexes (NuRD, N-CoR, Sin3a); the histone 3 lysine 4 methyltransferase (MLL) complex

and the LSD1 demethylase complex. All these data suggest a Nanog-dependent epigenetic regulation of distinct activated and repressed loci in pluripotent cells.

Oct4 is another key pluripotency factor in the transcriptional regulatory network in ESCs. Orphan nuclear receptor estrogen-related beta (Esrrb or Err2) was initially identified as an Oct4 partner [16]. Esrrb can recruit the Oct4/Sox2 heterodimer to the *Nanog* proximal promoter, and positively regulates Nanog expression. Together with other reports demonstrating that Nanog interacts with Esrrb and regulates its transcription [10,17], a feedback regulatory loop has been suggested wherein Oct4/Esrrb modulates Nanog expression and ESC pluripotency. An interactome centered by Oct4 was recently reported in three independent studies (van der Berg *et al* [18], Pardo *et al* [19], and Ding *et al* [20]). Because the levels of Oct4 are critical in controlling the undifferentiated state, ectopic expression of tagged Oct4 may affect the self-renewal capacity of ESCs [21]. Both Ding's and van den Berg's studies used ZHBTc4 mESCs, in which both *Oct4* alleles have been replaced and Oct4 expression is directed from a doxycycline-suppressible transgene [21], followed by re-introduction of either biotin- [20] or Flag-tagged [18] Oct4 in ZHBTc4 cells. There are 18 proteins identified consistently in three Oct4 interactomes, increasing the overall biological significance of those proteins. They are either involved in the NuRD (Chd4, Gatad2a, Gatad2b, Mta2, Mta3, Mbd3, Hdac1), SWI/SNF (also called BAF complex, Brg1, Baf155), or LSD1 (Lsd1, Rcor2) complexes, or they are individual TFs (Sall1, Sall4, Hcfc1, Hells, *etc*) playing important roles in ESCs. Interestingly, there is a high degree of consistency and numerous common transcriptional and epigenetic regulators revealed in both Oct4 and Nanog interactomes, which raises an important issue on how ubiquitous chromatin modifiers interact with pluripotency TFs to modulate an ESC-specific gene expression profile to maintain pluripotency.

In addition to the core pluripotency interactome pivoted by Oct4 and Nanog, Kim *et al* identified a distinct PPI network associated with c-Myc [22]. A notable finding from the Myc interactome is the interaction with the Tip60-p400 complex. An RNA interference (RNAi) screen revealed that Tip60-p400 is essential to maintain the pluripotent state, whereas inhibition of the components in the Tip60-p400 complex leads to differentiation [23]. Interestingly, the gene expression profiles upon Tip60 and p400 knockdown (KD) highly overlap with that of Nanog KD and are enriched for developmental regulators [23]. Together with the evidence that the Tip60-p400 complex is a Nanog [14] but probably not an Oct4 interactor (the Tip60-p400 complex was identified only in one of the three Oct4 interactome studies, and wasn't validated by co-immunoprecipitation [18]), a unique Nanog function of epigenetic regulation that differs from Oct4 is suggested.

ESC-specific protein complexes identified by AP-MS

Several structurally specialized protein complexes in ESCs were explored because AP-MS is able to identify individual protein components. A striking finding is the identification of the ESC-specific BAF complex (esBAF) [24], which has been shown to be critical for self-renewal and maintenance of the ESC state. Using a stringent AP condition, it was demonstrated that esBAF contains Brg1, BAF155, BAF60A, but not other known BAF components such as Brm, BAF170, and BAF60C. The esBAF complex was shown to

functionally interact with Oct4 and Sox2 and repress developmental genes by co-localizing genes with Oct4/Sox2 at the genomic loci, thus refining the core pluripotency circuitry [24,25]. BAF155 is also a component of the first Nanog interactome in mouse ESCs [10].

Another important ESC-specific protein complex is the Nanog/Oct4-associated deacetylase (NODE) complex identified by Nanog- and Oct4 AP-MS studies [15]. NODE lacks Chd4 and Mbd3, which are considered to be essential subunits of the canonical NuRD complex. However, both Chd4 and Mbd3 were later identified in the Oct4 interactome [18–20] as well as in the Nanog interactome ([14] and our unpublished data). Discrepancy of observations may be due to the fact that the NODE complex was purified by an endogenous antibody with low affinity and specificity, while the following studies used tagged Oct4 or Nanog for high affinity purification. Importantly, histone deacetylase activity is preserved in *Mbd3*$^{-/-}$ ESCs. KD of Mta1, a common subunit of NuRD and NODE complexes, resulted in upregulation of differentiation genes, which is distinct from that of Mbd3 KD [15]. Although it is suggested that both complexes are functionally important in ESCs, whether NODE is simply an experimental artifact, and if not, how the NODE and NuRD complexes assemble and distinctly interact with Nanog and Oct4, remains to be determined.

In addition, a nucleotide excision repair (NER) complex containing XPC, RAD23B, and CETN2 was uncovered as an Oct4/Sox2-dependent stem cell coactivator (SCC) complex, which is necessary for transcriptional activation of Nanog *in vitro* [26]. The SCC/XPC complex was isolated from a high-salt fraction of a multi-step chromatography purification procedure and identified by MS. It is worth noting that although interaction of Oct4 and SCC was validated in 293T cells by overexpression, a stable interaction in ESCs with endogenous levels of those proteins was unable to be reproduced [26]. Consistently, none of the subunits in the SCC complex was identified in the interactome studies of Oct4, suggesting that functional coactivator-activator interactions can often be weak and transient [27].

Pluripotency PPI network guides efficient somatic cell reprogramming (SCR)

Since the initial discovery of iPSCs induced by forced expression of "Yamanaka factors" Oct4, Sox2, Klf4, and c-Myc [2], the dynamics and molecular mechanisms of SCR have been extensively elucidated [28,29]. It is believed that forced expression of these genes perturbs various epigenetic processes leading to activation of the core pluripotency genes in reprogramming cells. Therefore, pluripotency interactome studies could strongly implicate how the ESC-like state is established and uncover many “necessities”, “drivers”, and “enhancers” of cellular reprogramming. For instance, the SCC/XPC complex is a requisite for reprogramming, as SCR efficiency is highly compromised in MEFs derived from XPC and RAD23B knockout mice [26]. Esrrb, an Oct4/Nanog interactor, was demonstrated as a driver of SCR capable of replacing Klf4 and c-Myc in the OSKM cocktail of reprogramming [30]. Moreover, combined overexpression of the Oct4/Nanog interactor esBAF components Brg1 and BAF155 has been reported to enhance OSK-induced reprogramming of fibroblasts [31]. Mechanistically, it was shown that Brg1 physically interacts with a conserved linker region between the POU-specific domain and the POU

homeodomain of Oct4. A point mutation at this linker region abolishes Oct4 activity during reprogramming, suggesting an important role of Oct4 in recruiting key epigenetic regulators to the genomic sites occupied by Oct4 [32].

The Mbd3/NuRD complex also associates with Oct4 and Nanog, as well as other important pluripotency factors such as Nac1, Sall4, and Zfp281 [10]. NuRD is required to modulate ESC heterogeneity by repressing the pluripotency genes; therefore it is considered a barrier of SCR [33,34]. Depletion of Mbd3 significantly enhances the reprogramming efficiency to a deterministic extend [35]. In stark contrast, a recent study by dos Santos *et al* [36] revealed an opposite functional contribution of NuRD complex to the reprogramming process. Depletion of Mbd3 abrogates epiblast stem cell (EpiSC) and pre-iPSC reprogramming but has a minimal effect on MEF reprogramming. Ectopic Mbd3 together with Nanog expression synergistically promotes EpiSC/pre-iPSC reprogramming [36]. The positive function of NuRD in reprogramming also finds its support from another study showing that the NuRD complex is recruited by Sox2 to repress mTOR expression and induce autophagy in promoting reprogramming [37]. It is currently unclear what the underlying cause for such discrepancies is, and thus future studies are needed to clarify the function of Mbd3/NuRD complex in the context of reprogramming. Interestingly, the Nanog-associated protein Zfp281 was shown to recruit the NuRD complex to the *Nanog* locus to restrict its expression in maintaining optimal self-renewal of ESCs and during late stages of the pre-iPSC to iPSC transition. KD of Zfp281 enhances the efficiency in this pre-iPSC reprogramming model by upregulating Nanog expression [38].

In an extended Nanog interactome study, association of Nanog and the ten-eleven translocation (TET) family of dixoygenase proteins uncovered a critical role of Nanog in recruiting Tet1 to the core pluripotency network [13]. Tet1-mediated demethylation is linked to the activation of pluripotency loci in the late phase of reprogramming, in accordance with the reorganization of the DNA methylation landscape at this stage [29]. Recently, a set of interactome studies on TET family proteins (Tet1/2/3) revealed a number of important interactors related to TET functions, such as the Sin3a-Hdac complex and O-GlcNAc transferase (Ogt) [39–41]. These interactions were also confirmed in the Sin3a interactome [42] and the Ogt interactome [43], respectively. Although discrepancy of observations was reported, it is believed that all TET family members interact with Ogt [39,44]. Ogt was shown to positively regulate SCR, while KD of Ogt decreases efficiency of OSK-driven MEF reprogramming [45]. Furthermore, Ogt interacts with and O-GlcNAcylates Oct4 and Sox2 post-transcriptionally. Modification of O-GlcNAc at T228 of Oct4 is functionally critical, as a point mutation at this site abolishes the effects of Oct4 in MEF reprogramming [45,46].

Perspectives

PPI is context sensitive and detection of interacting partners heavily dependents on the experimental procedures (i.e., sample treatment, salt concentration in AP) and the intrinsic abundance of interactors. Proteins can directly contact, or indirectly associate with each other through DNA or RNA. For instance, the Sox2-Oct4 interaction is DNA-dependent and must be stabilized by UV cross-linking [47,48]. Furthermore, it was reported that the long

intergenic noncoding RNAs (lincRNAs) play an important role in recruiting histone modifying complexes to genomic loci and mediating protein interaction [49,50]. Therefore, pretreating samples with benzonase is recommended only if the protein-protein interaction is investigated. Since an AP-MS experiment may not be able to discover all interactors of the bait protein, alternative methods can be used to complement the PPI network, such as high throughput yeast two-hybrid assays, domain-domain interactions, and protein microarray [51].

Given that most pluripotency factors are TFs, one urgent task for interactome studies is to ascertain transcriptional control in ESCs [52]. Together with the technique of chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq), interactomes interrogate how pluripotency TFs recruit co-activators/repressors and change the local epigenetic pattern and chromatin structure [53]. Genome-wide co-localizations of many interacting partners have been reported in ESCs, such as Nanog and its interactors Nac1, Dax1, and Zfp281 [53], Oct4 and Wdr5 [54], Tet1 and Sin3a [55], Tet1/2 and Ogt [39,41,43], and Hdac1/2 and NuRD [56]. Interestingly, ChIP-Seq also tells how a shared subunit functions in different protein complexes. For instance, Hdac1/2 are the subunits in both NuRD and Sin3a complexes. In mouse ESCs, Hdac1/2 showed a higher level of genomic co-localization with the NuRD than with the Sin3a complex (our unpublished data). NuRD represses the pluripotency TFs, while Sin3a was shown to positively regulate Sox2 and Nanog expression [57]. Therefore the Hdac activity mainly associated with the NuRD complex, a reprogramming barrier, is in line with the evidence that treatment of Hdac inhibitors accelerates SCR [58].

By now, most of the interactome studies on the pluripotency network are in mouse ESCs. Although the mouse is the most important organism for generating hypotheses in the field of stem cell biology, differences across species exist. Human ESCs are more similar to mouse EpiSCs in a primed state of pluripotency [59]. Obstacles in studying the human interactome include the high cost of maintaining human ESCs, lacking high affinity endogenous protein antibodies, and difficulty in genomic engineering of human cells. The advance of zinc-finger nuclease (ZFN) [60] and transcription activator-like effector nuclease (TALEN) [61] techniques comprise a powerful tool to manipulate human genes. Recently, the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system has shown great promise and flexibility for genetic engineering by multiplexed disruption and targeted integration of human genes [62,63]. High affinity pull-down is feasible by fusing epitope tags to the pluripotency factors at their endogenous loci.

As the functional players, protein expression and their interaction network delineate how they act individually and together in the processes of self-renewal and maintenance of pluripotency (Figure 2). Pluripotency protein interactome also provides a framework for exploring the new factors that may permit faithful reprogramming of somatic cells. Technically, further developments of interactome studies will continue to focus on increasing the sensitivity of AP-MS and reducing the required amount of proteins from a rare population of cells. A better understanding of the pluripotency machinery in ESCs awaits efforts on both systematic discovery of new interactors and sophisticated functional studies for those candidates.

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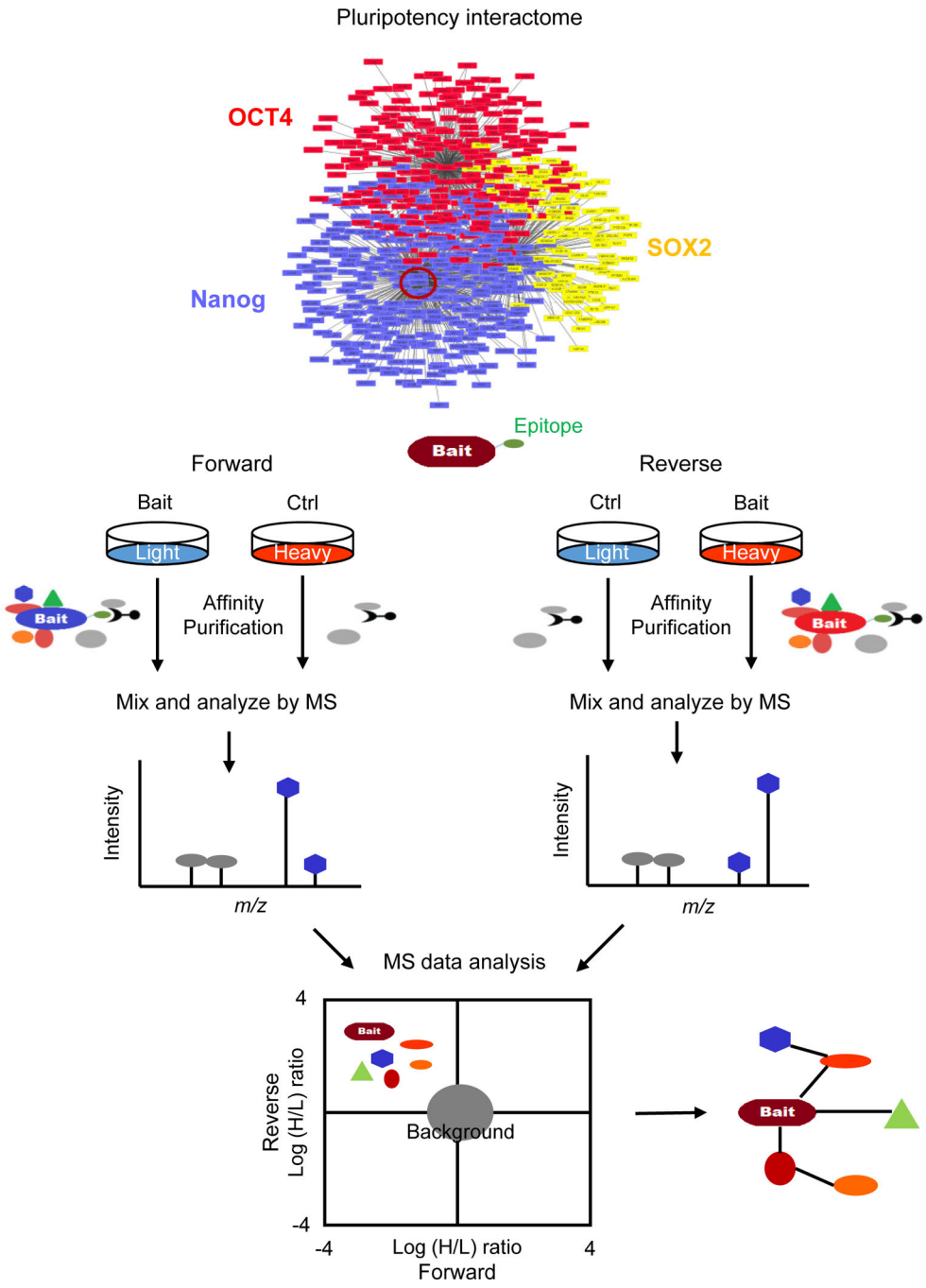

Figure 1.

Strategy of interactome study using affinity purification coupled with mass spectrometry (AP-MS). A bait protein (indicated as the red circle) in pluripotency interactome with an epitope (either endogenous or a tag-conjugated protein) can be specifically recognized and purified in AP. Salt concentration during purification is important. High salt concentration will remove relatively weak protein-protein interactions or protein-mediated indirect interactions. Endonuclease such as benzonase is used to destroy the DNA/RNA-mediated protein-protein interactions. The abundance of purified protein is determined by MS in two

strategies. In label-free method, protein abundance is quantified by counting the number of detected peptides. In stable isotope labeling method (shown in the figure), the lysates are combined together after AP in order to prevent potential heavy to light exchange of specific transiently interacting partners during the purification. Then protein abundance is quantified from the profiles of co-eluting light and heavy peptides, representing the relative amount of proteins from the bait- and control-AP, respectively. A biological replicate is recommended with label swapping (reverse experiment). Specific interactors by definition show a negative log heavy-versus-light (H/L) ratio in forward and a positive log (H/L) ratio in reverse experiments, whereas unspecific or background binders show a log (H/L) ratio close to 0 in both experiments. Finally, a protein-protein interaction network is constructed from the interactors surrounding the bait protein using a visualization program such as Cytoscape.

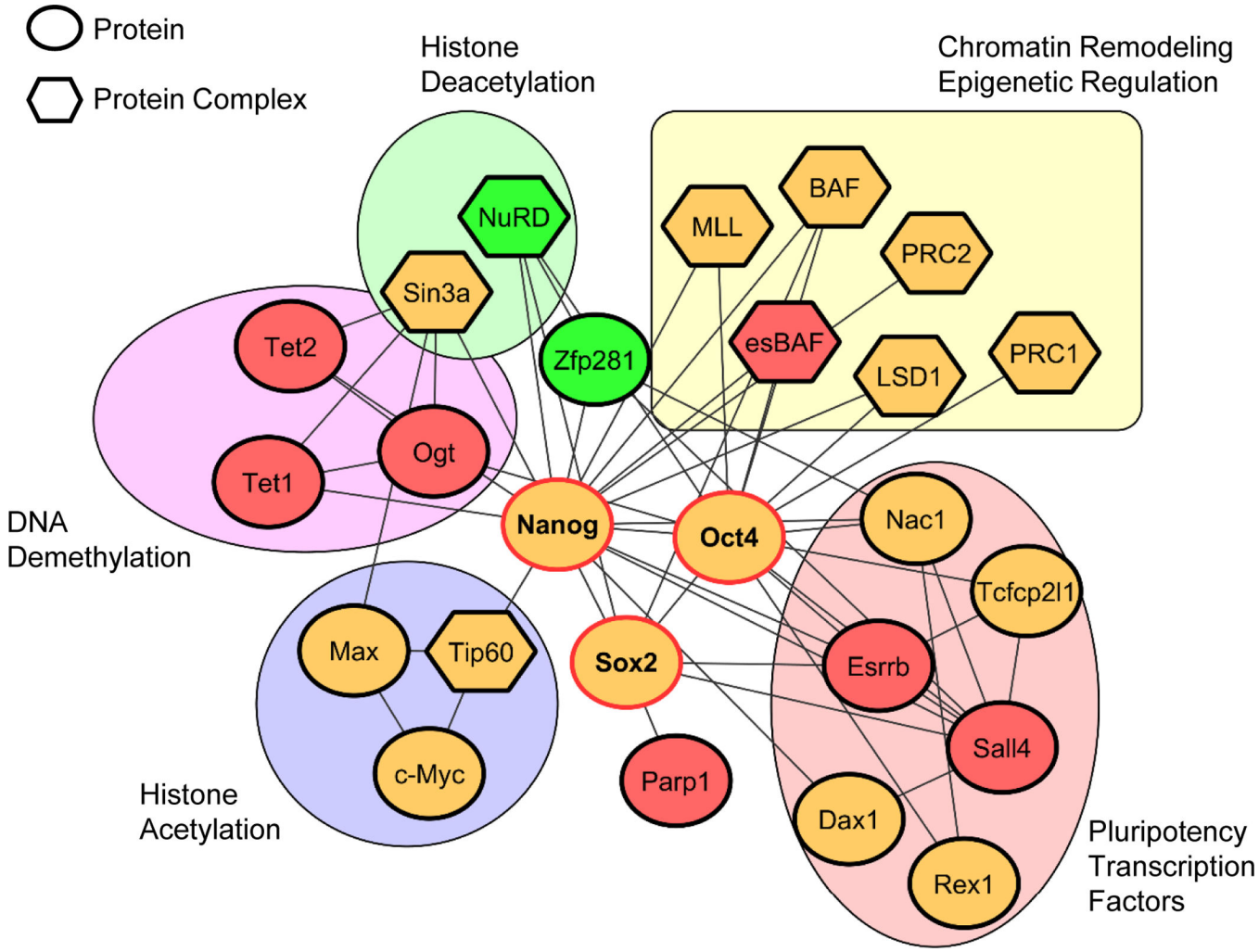

Figure 2.

An extended pluripotency interactome in ESCs. The network is an integrated view from multiple published interactome studies in mouse ESCs. Proteins (eclipses) and/or protein complexes (hexagons) are connected with solid lines, indicating physical associations with each other. The core pluripotency factors are indicated with bold text and red borders. Red-color filled shapes indicate the proteins that facilitate or replace one of the Yamanaka factors during somatic cell reprogramming. Green-color filled shapes indicate the barrier proteins of reprogramming. The proteins and protein complexes in the pluripotency interactome are classified into groups such as pluripotency transcription factors, histone acetylation, histone deacetylation, DNA demethylation, and other chromatin remodeling and epigenetic regulation complexes.

Table 1

Summary of the published interactome studies in ESCs.

Study	Bait proteins	Cell resource	Affinity Purification (AP) methods	Important interactors and its function
Wang *et al*, 2006 [10]	Nanog, Dax1, Nac1, Zfp281, Oct4, Rex1	Mouse ESCs	Biotin/streptavidin (Bio/SA) -AP	Survey study
van den Berg *et al*, 2008 [16]	Oct4	Mouse ESCs	Flag-AP	Esrrb; positively regulate Nanog expression
Liang *et al*, 2008 [15]	Nanog	Mouse ESCs	Endogenous antibody-AP	NODE complex; represses ESC differentiation
Ho *et al*, 2009 [24]	Brg1	Mouse ESCs	Endogenous antibody-AP	esBAF complex; a ES-sepcific BAF complex essential for self-renewal
Shen *et al*, 2009 [64]	Ezh1, Ezh2, Eed	Mouse ESCs	Double-step Flag-and Bio/SA-AP	Jmj (Jarid2); interacts with PRC2 complex and fine-tunes H3K27me3
Pasini *et al*, 2010 [65]	Suz12, Jarid2	AP-MS in Hela and 293T cells, validated in ESCs	Flag-AP (Suz12), double-step Flag- and HA-AP (Jarid2)	Jarid2; interacts with PRC2 complex and maintains H3K27me3
Kim *et al*, 2010 [22]	Myc, Max, Dmap1, Tip60, Gcn4, E2F4	Mouse ESCs	Bio/SA-AP	Survey study; defines three independent (core, PRC, and Myc) modules in ESCs
van den Berg *et al*, 2010 [18]	Oct4, Sall4, Dax1, Tcfcp2l1, Esrrb	Mouse ESCs	Flag-AP	Survey study
Pardo *et al*, 2010 [19]	Oct4	Mouse ESCs	Flag-AP	Survey study
Mallanna *et al*, 2010 [66]	Sox2	Early differentiating mouse ESCs	Flag-AP	Sox21; triggers differentiation
McDonel *et al*, 2012 [42]	Sin3a	Mouse ESCs	Flag-AP	Survey study
Ding *et al*, 2012 [20]	Oct4	Mouse ESCs	Bio/SA-AP	Survey study
Fidalgo *et al*, 2012 [38]	Zfp281	Mouse ESCs	Endogenous antibody-AP	NuRD complex; mediates Nanog autorepression
Gao *et al*, 2012 [67]	Sox2	Mouse ESCs	Flag-AP	Survey study; Smarcd1, represses ESCs differentiation
Lai *et al*, 2012 [68]	Sox2	Mouse ESCs	Flag-AP	Parp1; negatively regulates Sox2 in response to FGF signaling
Costa *et al*, 2013 [13]	Nanog	Mouse ESCs	Single-step Flag-AP, Bio/SA-AP, or Endogenous antibody-AP	Tet1; facilitates reprogramming
Gagliardi *et al*, 2013 [14]	Nanog	Mouse ESCs	Flag-AP	Survey study; domain-mapping of Nanog/Sox2 interaction
Chen *et al*, 2013 [41]	Tet2, Tet3	AP-MS in 293T cells, validated in ESCs	SA-binding peptide/SA-AP	Ogt; mediates histone 2B Ser112 GlcNAcylation
Shi *et al*, 2013 [40]	Tet1	Mouse ESCs	Endogenous antibody-AP	Ogt; modifies Tet1 by GlcNAC and positively regulates Tet1 expression
Vella *et al*, 2013 [43]	Ogt	Mouse ESCs	Double-step Flag-and Bio/SA-AP	Tet1; recruits Ogt to chromatin
Yakulov *et al*, 2013 [69]	β-Catenin	Mouse ESCs	Endogenous antibody-AP	Lsd1; is recruited by β-Catenin to repress Lefty1