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Myc orchestrates a regulatory network required for the establishment and maintenance of pluripotency

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Pluripotent stem cells (PSCs) are maintained by a complex regulatory network orchestrated by transcription factors, epigenetic modifiers and non-coding RNAs. Central to this regulatory network is the Myc family of transcription factors. Defining roles for Myc in PSCs has been problematic, but recently, a number of reports have provided insight in this area. An emerging picture now places Myc as a key regulator of the cell cycle, genomic maintenance and general metabolic activity in PSCs through its ability to directly regulate large numbers of target genes and more indirectly through control of microRNAs. One of Myc's main roles is to repress the activity of genes required for differentiation such as the endoderm master regulator, GATA6. The general mechanism by which Myc activates target genes is well understood but a remaining major challenge is to understand how it represses gene activity. Here we discuss potential mechanisms for how Myc establishes and maintains the pluripotent state and incorporate proteomics data that supports a model where Myc acts as part of a regulatory network with epigenetic modifiers.

Key words: myc, pluripotency, self-renewal, reprogramming, proteomics

Abbreviations: PSC, pluripotent stem cell; ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; LIF, leukemia inhibitory factor; dKO, double knockout

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Background

Embryonic stem cells (ESCs) are derived from pluripotent cells within the inner cell mass of blastocyst-stage embryos and like their in vivo counterparts, can differentiate into the three embryonic germ layers. Blastocysts also contain two extra-embryonic cell types; primitive endoderm which provides signaling support to the developing pluripotent epiblast and the trophectodermal layer which contributes to the placenta.[1-4] ESCs can be maintained for extended periods of time in culture as a stable, self-renewing population but without the appropriate balance of extrinsic signals, they lose pluripotency and differentiate into extra-embryonic cell types or germ layer lineages.[5-9] Perhaps the most important extrinsic signaling molecules required for maintenance of murine ESCs (mESCs) are the interleukin-6 family member cytokines, such as leukemia inhibitory factor (LIF).[10,11] A major role for LIF in mESCs is to maintain the expression of Myc transcription factors.[12] c-, N- and L-myc are a family of sequence-specific, basic helix-loop-helix transcription factors with well-established roles in embryonic development and cancer.[13-17] The discovery ~6 years ago linking Myc to LIFs role in maintaining mESC self-renewal[12] has stimulated much interest and insight into pluripotent cell biology. This report will summarize recent advances in this area and describes a model based on new data for how Myc participates in mechanisms relating to pluripotency and reprogramming.

Following the initial report that Myc is critical for self-renewal,[12] Yamanaka and colleagues demonstrated that Myc is also important for efficient reprogramming to an induced pluripotent cell (iPSC) state.[18,19]

Table 1. Percentage overlap of Myc target genes in mESCs expressed as pair-wise comparisons of the published data sets

	Chen	Kidder	Kim	Lin	Smith	Sridharan	
Chen	***100.00***	**19.94** *(38.66)*	**41.51** *(34.39)*	**12.16** *(11.54)*	**22.77** (41.23)	**27.62** *(37.82)*	2832[a]
Kidder		***100.00***	**51.61** *(22.06)*	**15.22** *(7.45)*	**33.65** *(31.43)*	**35.50** *(25.08)*	1459[a]
Kim			***100.00***	**13.53** *(15.50)*	**23.81** *(52.05)*	**29.73** *(49.15)*	3417[a]
Lin				***100.00***	**6.17** *(11.78)*	**8.36** *(12.06)*	2980[a]
Smith					***100.00***	**51.86** *(39.23)*	1562[a]
Sridharan						***100.00***	2065[a]

Values represent the overlap as % of the total number of target genes in six genome-wide location analysis studies.[20-25] [a]Total number of target genes from each study.

Myc Target Genes in Pluripotent Cells

To determine how Myc regulates pluripotency, several ChIP-Chip and ChIP-Seq studies have been performed, resulting in the identification of a large number of in vivo target genes.[20-25] Although over 8,000 target genes have been identified by these studies, only ~19 are common to all six. When different pair-wise comparisons of published Myc target genes are made, the overlap ranges from 6 to 52% (**Table 1**). Despite the low degree of overlap in Myc targets between different studies, many have been validated independently by ChIP-qPCR assays. Overall, these results indicate that individual studies directed towards global in vivo target gene identification are not particularly exhaustive and that multiple independent approaches are required to obtain a complete picture. At least some of the variations described can be accounted for by differences in gene expression signatures between different cell lines, differences in the platform technology employed and the bioinformatics analysis used. The routine introduction of deep sequencing-based approaches (Chip-seq and RNA-seq) should circumvent some of these issues. When the criteria used for comparison of data between different studies are relaxed and data from only four studies[20-22,25] are included in the analysis, 314 common Myc targets are identified (**Fig. 1A**). Functional annotation of these target genes shows significant enrichment in processes relating to cell cycle control, DNA replication and repair, gene expression and metabolism ($p < 0.001$, **Fig. 1B**). These high-confidence targets therefore form the 'core' Myc gene-target network that establishes and maintains PSCs.

How does Myc Regulate Pluripotency?

For some time, Myc has been associated with inhibition of cellular differentiation, especially in the context of cancer biology.[16,26] Two reports recently showed that simultaneous genetic inactivation of c-MYC and N-MYC in ESCs and iPSCs resulted in rapid loss of PSC colony morphology and pluripotent marker expression.[15,25,27] Rather than differentiating randomly into different lineages, we found that c-, N-MYC double knockout (dKO) cells preferentially formed primitive endoderm.[25] The mechanism for this involves de-repression of the endoderm master regulator gene, GATA6. These findings indicate that under self-renewing conditions, differentiation towards endoderm is blocked by Myc-dependent repression of GATA6, adding yet another level of complexity to the mechanism by which Myc maintains the pluripotent state. Analysis of the GATA6 locus in mESCs[28,29] reveals it to be bivalently marked (H3K4me3 and H3K27me3; **Fig. 2A**) consistent with it being inactive but poised for activation. Nanog, a known repressor of GATA6,[30,31] also binds the regulatory region,[32] but it is unclear how and if it works in conjunction with Myc. Since Nanog and Myc repress GATA6, this raises an interesting potential link between two well-established regulators of pluripotency.

Besides a differentiation blockade being a critical part of Myc's role in maintaining pluripotency, several lines of evidence indicate that its ability to control the cell cycle is also important. This is suggested by decreased rates of cell division and a remodeling of the cell cycle following Myc inactivation.[25] As mentioned previously, numerous cell cycle regulators including cyclins and cyclin dependent kinases are Myc target genes (**Fig. 1B**), providing a potential mechanism that could explain cell cycle changes following loss of Myc activity. Similar to the situation in various cancer cells, the mir-17-92 cluster is actively expressed in ESCs and is regulated at the transcriptional level by c- and N-myc.[15,20,25] In PSCs, the cluster is marked by the active H3K4me3 and H3K36me3 modifications[29] (**Fig. 2B**). The mir-17-92 locus produces miRNAs that target many cell cycle regulators such as transcripts for E2F transcription factors, cyclins and members of the retinoblastoma (Rb) tumor suppressor family.[33,36] Our recent findings indicate that in PSCs, mir-17-92 targets cell cycle regulators such as RB2/p107,[25] introducing a post-transcriptional level of cell cycle control regulated by Myc. By extension, during cellular reprogramming it is likely that Myc directs the establishment of an ESC-like cell cycle program by elevating global CDK activity and suppressing tumor suppressor activity.[37] To support this, the cell cycle inhibitor p21 inhibits iPSC generation[38] and conversely, knockdown of p21 accelerates cell division which leads to faster cellular reprogramming of differentiated cells by Oct4, Sox2, Klf4 and c-myc.[39]

Identification of Interacting Proteins as a Guide to Myc's Role in Pluripotent Cells

Myc's role as a prolific regulator of gene expression in PSCs can be further understood by defining the types of protein complexes it is assembled into. To characterize Myc-containing complexes in mESCs, we used a mass spectrometry-based proteomics approach. Antibody-coupled

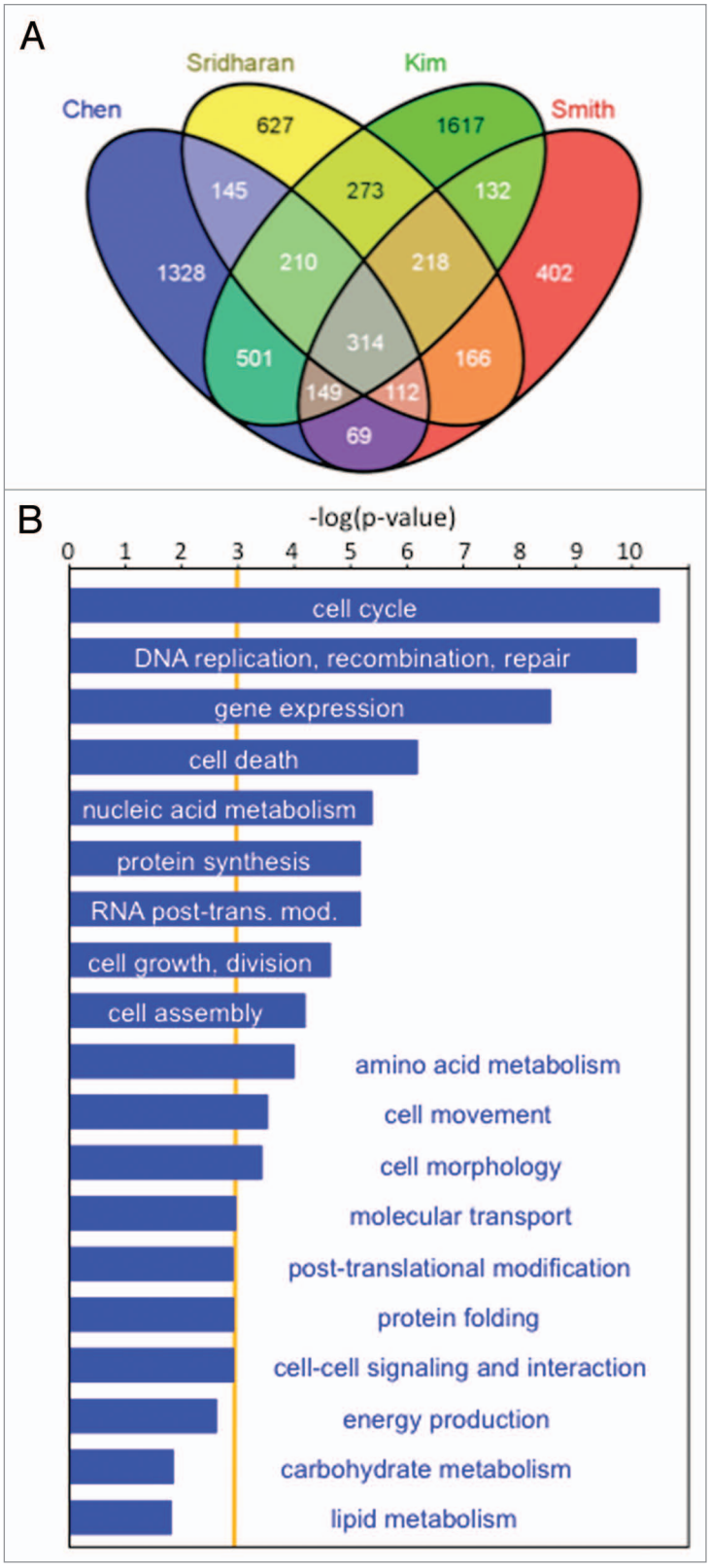

Figure 1. In vivo target genes for c-myc in mESCs. (A) Venn diagram constructed as in reference 53 showing the proportion of Myc target genes common to four genome-wide ChIP studies.[20-22,25] 314 target genes are found to be c-myc target genes in all four studies. (B) Functional annotation of target genes found in all four datasets indicates enrichment primarily for regulators of the cell cycle, gene expression, genomic maintenance and metabolic activities. Data were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). The orange line represents the cut-off for statistical significance (p-value-0.001). The x-axis denotes—(log) significance where longer bars represent greater enrichment of specified functional categories.

magnetic beads were used to isolate Myc complexes from ESC lysates, followed by liquid chromatography-tandem mass spectrometry. Non-specific interacting proteins were identified by subtractive methods using a Myc null cell line.[40] The resulting list of 26 proteins that specifically immunoprecipitated with Myc included transcriptional regulators and epigenetic modifiers (**Fig. 3A**). As expected, Myc's well-characterized binding partner, Max, was identified in immunoprecipitates. Other known interacting proteins identified in this analysis include Ruvbl1, Ruvbl2 (Tip48, Tip49), Hdac1 and Top2a.[41,42] The DNA helicases Ruvbl1 and Ruvbl2 are components of multiple chromatin remodeling complexes[43,44] and RNAi mediated knockdown of either factor results in loss of the characteristic ESC colony morphology.[45] Interestingly, Ruvbl1, Ruvbl2 and Hdac1 are also components of the repressive NURD complex that functions in nucleosome remodeling.[46] Previous studies have shown that the NURD complex component MBD3 is required for pluripotency maintenance.[47] Recruitment of the repressive histone deacetylase activity associated with NURD[46] is therefore one mechanism by which Myc could act to block expression of specific target genes.

Another epigenetic regulator with repressive function, Lsd1 (Kdm1a), was also found in Myc-immunoprecipitates (**Fig. 3B**). Lsd1 functions as a histone demethylase and its action on dimethylated lysine 4 of histone H3 results in gene repression.[48] Lsd1 and Hdac1 are components of the CoREST co-repressor complex that cooperates with REST to inhibit neural specification factors in non-neural lineages,[49] and Myc has recently been found to interact with Rcor3, another corepressor of Rest.[50] Recently, Lsd1 has also been shown to interact with other pluripotency regulators such as Nanog, but perhaps more significant are observations that loss of Lsd1 activity results in

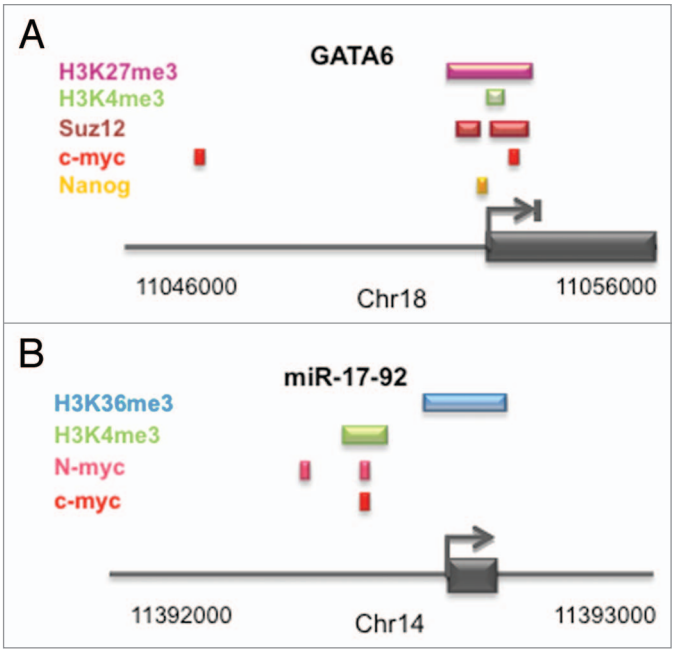

Figure 2. Epigenetic control of positively and negatively regulated Myc target genes in mESCs. (A) Schematic of the bivalently marked GATA6 gene[29] showing the upstream regulatory region, positional binding of transcriptional regulators and histone modification status in the region.[20,25,29] The GATA6 gene is repressed in pluripotent cells. (B) Schematic of the transcriptionally active murine miR-17-92 cluster and its upstream regulatory region showing binding of c-myc and N-myc together with active histone modifications.[20,25,29]

increased apoptosis and impaired differentiation of ESCs.[51,52] The identification of Lsd1 as a Myc-interacting protein suggests that Myc may repress target genes through a CoREST-dependent mechanism in PSCs.

Another recent proteomic study identified an additional set of Myc interacting proteins in PSCs including Trrap, Ep400, Dmap1, Brd8, Epc1 and Epc2.[42] These proteins are members of the NuA4 acetyltransferase complex which appear to be important for maintenance of PSCs.[45] Altogether, these proteomic studies indicate that Myc regulates PSCs as a component of different regulatory protein complexes with known roles in gene repression and activation.

Materials and Methods

Myc-interacting proteins were identified using nuclear extracts from mESC lines expressing 9E10-tagged c-myc, or a c-myc null ESC line[40] as the control. mESCs were allowed to swell in 10 mM HEPES pH 7.4, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and protease inhibitors. NP-40 was added to 1.6% final v/v, followed by brief vortexing to disrupt the cellular membrane and centrifugation to pellet nuclei followed by removal of the cytoplasmic supernatant. Nuclear proteins were extracted with 20 mM HEPES pH 7.4, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and protease inhibitors. Extracts were subjected to immunoprecipitation with tosyl-activated magnetic beads (Invitrogen) coupled to affinity-purified 9e10 monoclonal antibody according to the manufacturer's instructions. Complexes were then eluted with glycine (pH 2.5) followed by neutralization with Tris-HCl pH 8.8. In-solution tryptic digests were desalted then analyzed by nLC-MS/MS on a linear ion-trap (LTQ, ThermoFisher) as previously described in reference 54. Data was collated and filtered to obtain a 1% false discovery rate at the protein level using the ProteoIQ software package (BioInquire) and a reverse database to calculate FDR. An immunoprecipation from a cell line not expressing c-myc was used as a negative control and filtered at 5% false discovery (less stringent than the experimental sample). Proteins that were identified by at least two peptides in the experimental sample in at least two of the three replicates but were not identified in the negative control sample were considered high-confidence interacting proteins. Proteins that were identified with three times more coverage in the experimental sample (at 1% FDR) over the negative control (at 5% FDR) generated a list of putative interacting proteins that were further considered.

Conclusions

Although Myc is essential for maintenance of pluripotency, its function in PSCs has until recently been unclear. Recent work has identified a diverse range of in vivo target genes that point towards roles in gene regulation associated with cell cycle control, DNA replication/repair and metabolic control. Importantly, Myc seems to maintain the pluripotent state by blocking differentiation pathways, as shown by its direct effect on the GATA6 gene. Further studies will address the question of whether Myc blocks differentiation along other pathways. The finding of active and inactive target genes identified in PSCs points towards roles for Myc in gene activation and repression. This is consistent with the regulatory complexes Myc assembles into, with activating and repressive activities.

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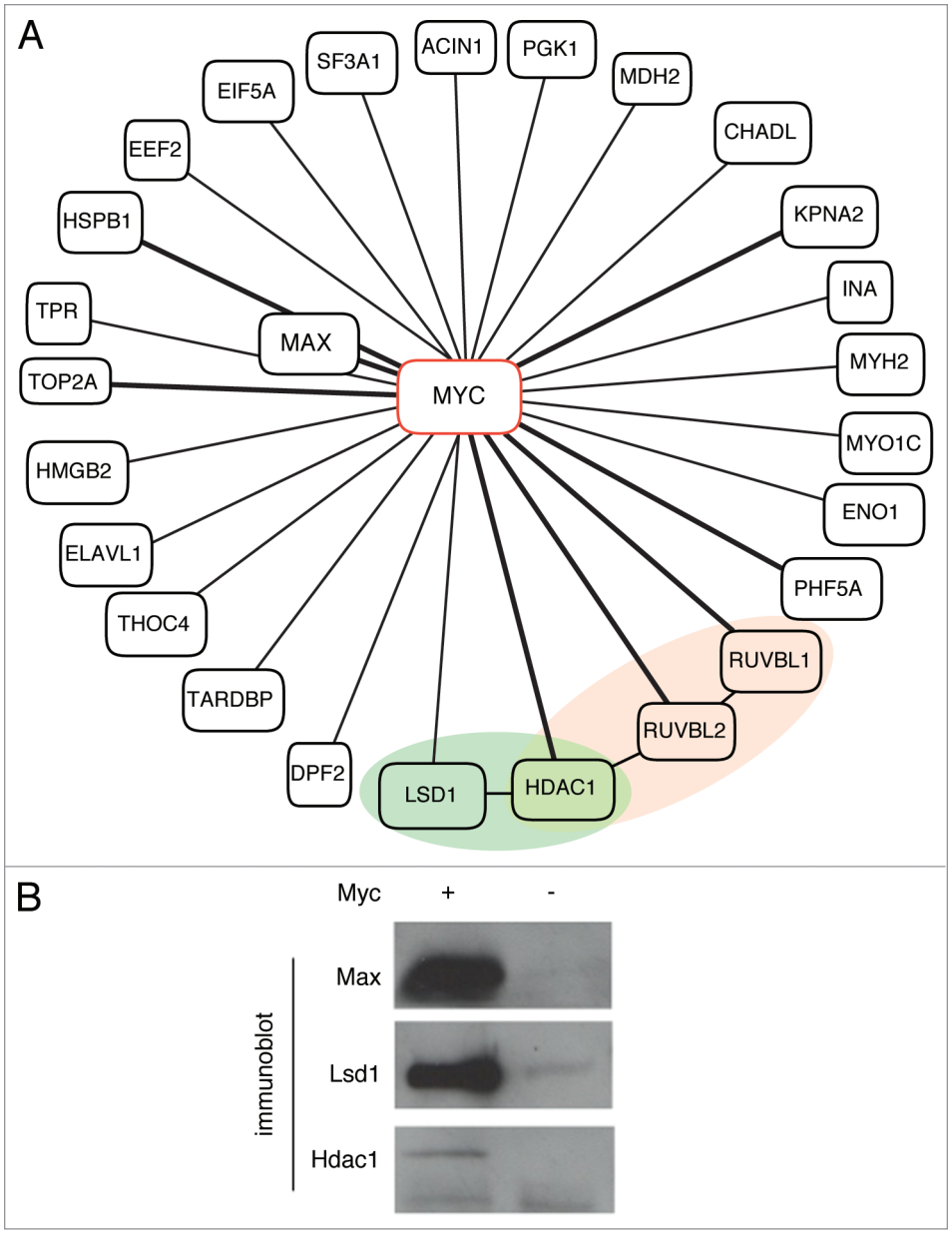

Figure 3. The Myc protein interaction network in ESCs. (A) Diagrammatic representation of Myc interacting proteins identified by affinity purification coupled with mass spectrometry. Previously identified protein partners[41,50] are joined by bold connectors. Myc interacting protein members implicated in transcriptional repression are shaded. The protein network is based on that generated through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). (B) c-myc assembles into complexes with members of the coREST co-repressor complex in pluripotent cells. Co-immunoprecipitation analysis with antibody coupled magnetic beads validates the interaction of c-myc with Lsd1 and Hdac1 in a cell line expressing Myc (AB2.1,$^{6x9e10\text{-}c\text{-}myc}$; +) and not the control Myc null cell line (AB2.1$^{c\text{-}myc-/-}$; -). Max is used as a control.

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