Roles for MYC in the Establishment and Maintenance of Pluripotency

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MYC and MYCN have been directly implicated in the transcriptional regulation of several thousand genes in pluripotent stem cells and possibly contribute to the activity of all transcribed genes. Control of transcription by a pause-release mechanism, recruitment of positive and negative epigenetic regulators, and a general role in transcriptional amplification have all been implicated as part of the broad, overarching mechanism by which MYC controls stem cell biology. As would be anticipated from the regulation of so many genes, MYC is involved in a wide range of cellular processes including cell-cycle control, metabolism, signal transduction, self-renewal, maintenance of pluripotency, and control of cell fate decisions. MYC transcription factors also have clear roles in cell reprogramming and establishment of the pluripotent state. The mechanism by which MYC accomplishes this is now being explored and promises to uncover unexpected facets of general MYC regulation that are likely to be applicable to cancer biology. In this work we review our current understanding of how MYC contributes to the maintenance and establishment of pluripotent cells and how it contributes to early embryonic development.

Well before MYC was implicated in the establishment and maintenance of pluripotency, it was known as a potent oncogene with roles in transcriptional regulation of metabolism, differentiation, cell lifespan, cell cycle, and cell size control. These functions are all generally relevant to the maintenance and establishment of pluripotent stem cells. Despite this, however, defining the precise mechanism by which MYC functions has been problematic and has led to much confusion. The following discussion will focus on our current understanding of how MYC functions in early embryonic de-

velopment, maintenance of stem cell identity, and in somatic cell reprogramming.

MYC AND MYCN ARE FUNCTIONALLY REDUNDANT IN EARLY EMBRYONIC DEVELOPMENT

The MYC family of basic helix-loop-helix leucine zipper transcription factors consists of MYC, MYCN, and MYCL. DNA binding of MYC family members usually requires heterodimerization with MAX (Myc-associated factor X) through their respective leucine zipper do-

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mains (Blackwood and Eisenman 1991; Blackwell et al. 1993). Knockout studies in mice show that no single *MYC* family member, or *MAX*, is essential for early development and that *MYCL* is completely dispensable for all embryogenesis (Hatton et al. 1996; Shen-Li et al. 2000). Individual knockout of *MYC*, *MYCN*, or *MAX* in mice is inconsequential until around the mid-gestation stages of development. The expression of *MYC* and *MYCN* overlaps considerably before gastrulation, explaining the absence of clear developmental defects in single knockouts in pre- and early postimplantation embryos. At this time point, *MAX* expression becomes essential as knockout mice at ~5.5–6.5 dpc (days postcoitum) cease development (Shen-Li et al. 2000). As primitive tissues begin to form, the expression patterns of *MYC* and *MYCN* change. In midgestation development, when *MYC* and *MYCN* expression becomes nonoverlapping, tissue-specific defects are observed following deletion of *MYC* or *MYCN*. *MYC*^{-/-} embryos, for example, are growth retarded and die before ~10.5 dpc with cardiac, neural, and vascular abnormalities (Davis et al. 1993; Baudino et al. 2002). *MYCN*^{-/-} embryos die between 10.5 and 12.0 dpc with defects in several of the visceral organs including lung, heart, liver, gut, and the peripheral and central nervous systems (Charron et al. 1992; Stanton et al. 1992; Sawai et al. 1993).

In a key experiment to evaluate the functional redundancy of *MYC* and *MYCN* in a developmental context, Malynn and coworkers engineered a mouse in which *MYC* coding sequences were substituted with *MYCN*. Mice expressing *MYCN* under transcriptional control of the *MYC* locus grew to adulthood, reproduced, and generated fertile offspring with only mild skeletal muscle defects (Malynn et al. 2000). This report is often used as evidence to argue that *MYC* and *MYCN* are functionally redundant but it is possible that in disease pathogenesis such as cancer, the two family members may have nonequivalent functions (Kawauchi et al. 2012; Roussel and Robinson 2013).

Several reports also implicate key roles for *MYC* family members in the maintenance, expansion, and differentiation of stem cell popu-

lations including pluripotent stem cells, hematopoietic stem cells, and keratinocyte stem cells (Gandarillas and Watt 1997; Laurenti et al. 2008; Wilson et al. 2008). Embryonic stem cells (ESCs) isolated from mice lacking either *MYC* or *MYCN* are capable of long-term self-renewal and remain pluripotent (Malynn et al. 2000), consistent with other studies showing that *MYC* performs redundant roles in peri-implantation development. Simultaneous deletion of floxed *MYC* and *MYCN* alleles, however, destabilize the pluripotent state resulting in differentiation toward endoderm and mesoderm (Smith et al. 2010; Varlakhanova et al. 2010). A similar phenomenon occurs in hematopoietic stem cells, in which loss of either *MYC* or *MYCN* expression is largely inconsequential, whereas deficiency of both is lethal (Laurenti et al. 2008). In ESCs ectopic reexpression of *MYC* or *MYCN* restores pluripotency (Smith et al. 2010), supporting the idea that *MYC* and *MYCN* perform redundant roles in maintaining pluripotent stem cell identity.

MYC PROMOTES THE MAINTENANCE OF PLURIPOTENT STEM CELLS

Pluripotent stem cells (PSCs) divide symmetrically, generating two equivalent daughter stem cells at each mitosis (Zwaka and Thomson 2005). These cells retain the ability to self-renew over long periods of time and the capacity for differentiation toward multiple lineages associated with the three embryonic germ layers. Under feeder-free conditions, murine PSCs are typically cultured in the presence of an interleukin-6 cytokine family member, such as leukemia inhibitory factor (LIF). LIF serves to maintain pluripotency by binding gp130/LIF receptor heterodimers, resulting in phospho-activation of the STAT3 transcription factor (Ernst et al. 1996; Niwa et al. 1998). STAT3 then activates target genes involved in cell-cycle control, immortalization, and suppression of differentiation. The first evidence pointing toward *MYC* playing a role in pluripotency came from the work of Cartwright and coworkers in 2005 (Cartwright et al. 2005). Here, *MYC* was shown to be a direct downstream transcriptional target

of LIF/STAT3 signaling and that sustained *MYC* expression could alleviate the requirement for LIF over extended periods of time. Numerous regulatory proteins are responsible for modulating MYC degradation (discussed in Farrell and Sears 2013) and one notable feature of MYC protein in PSCs is its unusually long half-life. Pulse-chase experiments show that MYC stability in PSCs is comparable to oncogenic mutants, such as T58A. This enhanced stability requires canonical PI3K signaling to be active so that GSK-3 β activity is suppressed. Once LIF signaling is disrupted in mouse ESCs (mESCs), early differentiation is triggered by the collapse of PI3K activity, the activation of GSK-3 β and the degradation of MYC. The down-regulation of MYC and MYCN levels is dependent on loss of both STAT3-dependent transcriptional activation and enhanced MYC protein turnover. Coordination of these events is critical for the transition from the self-renewing, pluripotent state to that of an early, lineage-committed cell.

Although the individual inactivation of *MYC* or *MYCN* has no discernible effect on plu-

ripotency, their simultaneous loss destabilizes PSCs and results in differentiation toward primitive endoderm and mesoderm lineages (Smith et al. 2010). A key target of MYC in PSCs is the endoderm master regulator, *GATA6*. Transcriptional repression of *GATA6* in PSCs requires MYC, and, following loss of MYC activity, *GATA6* levels increase and cells differentiate toward primitive endoderm. *MYC* also induces a number of miRNA targets that inhibit PSC differentiation (Lin et al. 2009a). One such target is the *mir-17–92* cluster (Smith et al. 2010). These miRNAs maintain the rapid cell-division cycle of PSCs by elevating the activity of cyclin-Cdk complexes and by limiting the activity of tumor suppressors, such as members of the retinoblastoma (RB) family and potentially p16^{INK4a}. The MYC/MAX network has also been shown to coordinate with BMP-4 signaling to activate phosphatases that repress mitogen-activated protein kinase (MAPK) signaling (Fig. 1) (Hisida et al. 2011; Li et al. 2012; Chappell et al. 2013). This is significant because activation of MAPK signaling by fibroblast growth factors

Figure 1. External factors stimulate internal pathways, which cooperate to maintain PSCs. LIF/STAT signaling through MYC and/or BMP signaling through SMAD up-regulate dual-specificity phosphatases (DUSPs) to suppress FGF-stimulated ERK activity, thereby blocking differentiation.

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(FGFs) is known to increase the susceptibility of PSCs to differentiation signals (Kunath et al. 2007). Loss of *MYC* or *MAX* in PSCs leads to the down-regulation of ERK phosphatases, DUSP2 and DUSP7, stimulating MAPK signaling and resulting in differentiation (Chappell et al. 2013). The observation that differentiation can be blocked by a small-molecule inhibition of MEK provides an explanation for how PSCs are maintained by MAPK/MEK inhibition in LIF-depleted media (Ying et al. 2008). Under these conditions, self-renewal is not dependent on elevated *MYC*/*MYCN* levels (Marks et al. 2012) and implies that a major role for *MYC*/*MYCN* in PSCs is linked to its role as a negative regulator of MAPK signaling.

COMPLEXITIES OF MYC-DEPENDENT TRANSCRIPTIONAL REGULATION IN PLURIPOTENT CELLS

MYC clearly plays a role in gene activation as part of its overall role in maintaining pluripotency, and this activating function is typically associated with its ability to heterodimerize with *MAX* at enhancer boxes (E-boxes; 5'-CA CGTG-3') (Blackwood and Eisenman 1991). Recently, *MYC* has been proposed to regulate all actively transcribed genes in various cell types, including PSCs. In effect, this “universal amplifier” model implicates *MYC* as a nonlinear amplifier of transcription for all active genes, whereby the amount of *MYC* bound near a gene's transcription start site is correlated with transcriptional activity. Studies in PSCs indicate that *MYC* acts at transcription start sites by recruiting factors that release stalled RNA polymerase II complexes, promoting productive transcriptional elongation (Fig. 2A) (Rahl et al. 2010; Lin et al. 2012; Nie et al. 2012). Although this activity is certainly important for enhancing cellular growth and proliferation, the issue of to what extent this activity accounts for the significance of *MYC*'s contribution to pluripotent biology is still unresolved.

Other studies suggest that *MYC* has important roles that are inadequately encompassed by the universal amplifier model. For example, *MYC* interacts with a diverse range of epigenetic

modifiers to promote open, dynamic chromatin that is accessible to the transcription machinery (Gaspar-Maia et al. 2011; Orkin and Hochedlinger 2011). *MYC* interacts with TRRAP to recruit components of the GCN5 and NuA4 histone acetyltransferase (HAT) complexes to target genes (McMahon et al. 2000; Lin et al. 2009b). In some cases, this function appears to be important for maintenance of ESC identity, and for the establishment and maintenance of euchromatic chromatin in PSCs by directing H3 and H4 acetylation (Fig. 2A) (Frank et al. 2001; Cotterman et al. 2008). Furthermore, transcriptional repression is also an important *MYC* function unaccounted for by the universal amplifier model. Besides the repression of *GATA6* (Fig. 2B) (Smith et al. 2010), *MYC* also represses other developmentally important genes, such as the *HOX* cluster, in collaboration with the POZ domain/Zn-finger transcription factor MIZ-1 (Varlakhanova et al. 2011). *MYC* also interacts with components of the NuRD repressive complex such as HDAC1, RUVBL1, RUVBL2, and also components of the CoREST complex such as LSD1 (Smith et al. 2011). Interestingly, *MYC* has been shown in *Drosophila* to negatively autoregulate its own transcription through a polycomb-dependent mechanism (Khan et al. 2009). It remains unclear whether polycomb complexes broadly play a role in *MYC*-mediated transcriptional repression, but it is interesting to note that ~10% of *MYC*-binding sites are co-occupied by the polycomb subunit SUZ12 and by the H3K27 trimethyl mark (Lin et al. 2009b).

Over the last several years, ChIP-Chip and ChIP-Seq have been critical for defining gene regulatory networks in PSCs that are controlled by core pluripotency factors, such as OCT4 and NANOG (Chen et al. 2008; Kidder et al. 2008; Lin et al. 2009b; Sridharan et al. 2009; Kim et al. 2010; Smith et al. 2011). Similarly, these genome-wide binding studies have determined that *MYC* binds, and possibly regulates, the transcription of at least 8000 genes in PSCs (Smith et al. 2011). Despite *MYC* being critical for pluripotency, it clearly regulates a set of genes distinct from those regulated by core pluripotency factors. This set of genes termed the “*MYC* module” suggests a somewhat contrasted

Figure 2. (A) LIF/STAT signaling stimulates transcription of MYC, which then heterodimerizes at E-boxes with MAX and amplifies transcription of active genes. MYC promotes productive transcriptional elongation by releasing stalled RNA polymerase II at transcription start sites. MYC promotes open, accessible chromatin by recruiting components of the MLL histone methyltransferase complex, such as GCN5, and by interacting with HATs, such as the NuA4 complex, which is important for PSC identity and maintenance of euchromatic chromatin. (B) MYC has been shown to act as a transcriptional repressor of developmentally important lineage specifiers, such as *GATA6* and *HOX* genes, by interacting with MIZ-1 and components of the NuRD repressive complex such as HDAC1, RUVBL1, RUVBL2, and also components of the CoREST complex such as LSD1.

role for MYC compared with the universal amplifier model described above (Chen et al. 2008; Kim et al. 2010). In this model, MYC functions to regulate only a subset of genes, many of which are distinctly important for the maintenance of self-renewal and pluripotency. Interestingly, the MYC module can also be identified in cancer

cells, implying that MYC's role in pluripotency is related to its function in tumorigenicity (Kim et al. 2010; Rothenberg et al. 2010). Analysis of the MYC module reveals that MYC functions to regulate gene networks important for self-renewal such as growth, metabolism, and cell-cycle progression. Closer examination reveals that

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specific targets associated with pluripotency have been identified. For example, MYC binds and regulates the *SOX2* gene (Lin et al. 2009b). This is significant because minor fluctuations in *SOX2* expression have been shown to have a dramatic effect on PSC maintenance (Niakan et al. 2010). MYC also induces expression of the polycomb repressive complex components both directly, as in BMI1 (Guney et al. 2006), and indirectly as in EZH2 (Kaur and Cole 2013), which can then repress lineage-specifying genes downstream (Boyer et al. 2006). MYC also regulates self-renewal and pluripotency by transcriptionally activating microRNAs that inhibit differentiation and promote cell division (Lin et al. 2009a). One example is the *mir-17-92* cluster that inhibits expression of cell-cycle genes such as *E2F1*, *CCND1*, and *RB2* (Smith et al. 2010).

ESTABLISHMENT OF PLURIPOTENCY

Shinya Yamanaka's Nobel Prize winning work shows that forced expression of four transcription factors, OCT4/SOX2/KLF4/MYC (OSKM), was sufficient to reprogram a differentiated somatic cell to an induced pluripotent stem cell (iPSC) state (Takahashi and Yamanaka 2006). This discovery complemented previous work that showed the concept of reprogramming by nuclear transfer technologies (Gurdon et al. 1958; Gurdon and Uehlinger 1966) but in addition, created new opportunities for human disease modeling, drug screening, and regenerative medicine. Many variations to Yamanaka's original approach have now been described (Buganim et al. 2012), including the substitution of MYC with other factors including the histone deacetylase (HDAC) inhibitors valproic acid (Huangfu et al. 2008) and trichostatin A (Araki et al. 2011). Presumably, these factors mimic the effects of MYC in the reprogramming process by promoting the formation of euchromatin. Reprogramming in the absence of ectopically expressed MYC, however, is generally less efficient and delayed relative to when MYC-containing reprogramming cocktails are used (Nakagawa et al. 2008). As mentioned above, MYC can be omitted from reprogramming cocktails

but questions have been raised as to the quality of reprogrammed cells generated under different conditions. One recent report provides evidence that MYC is essential to generate fully reprogrammed cells and that this is dependent on its ability to recruit HAT complexes to target genes (Araki et al. 2011). The use of wild-type MYC in clinical settings to generate PSCs is currently not favored because of its potential to deregulate cell proliferation (Okita et al. 2007) but one group recently showed that the ability of MYC to reprogram may be independent of its role in transformation (Nakagawa et al. 2010). They showed that utilizing MYCL, or transformation defective MYC mutants, leads to a decrease in tumorigenicity. This opens up the possibility that other forms of MYC may have utility; however, because MYCL has been associated with some tumors, this phenomenon demands further investigation.

Of the four individual Yamanaka factors, MYC has the most potent effect on being able to establish a PSC-like gene expression profile and appears to exert its effects early in the reprogramming process (Mikkelsen et al. 2008; Sridharan et al. 2009). A novel mechanism was recently described demonstrating that MYC functions as an enhancer of OSK binding to inaccessible chromatin in the early stages of reprogramming (Soufi et al. 2012). OSK serve as "pioneer factors" by engaging enhancers of genes required for the establishment of pluripotency. MYC then binds modified enhancer boxes nearby and enhances the reprogramming process by a mechanism that is yet undetermined, but likely involves recruitment of chromatin-opening cofactors. At later stages of reprogramming, the combined effects of OSKM render chromatin more accessible around key pluripotency genes and colocalization of OSKM is lost as each factor resolves to their respective binding sites (Fig. 3). This novel function in early reprogramming appears to be independent of transcriptional regulation, indicating that the universal amplifier model, discussed above, may be more applicable to MYC's role in stem cell maintenance than to establishment of pluripotency. Interestingly, this permissive role of MYC may be similar to the way in which aber-

Figure 3. OSK act as “pioneer factors” in reprogramming. MYC stabilizes and enhances binding of OSK to regulatory regions in the first 48 hours. This involves transient binding of MYC to noncanonical E-boxes, resulting in the reinforcement of OSK binding. This promotes euchromatin formation that renders promoters accessible in fully reprogrammed cells. MYC, OCT4, and KLF4 can then bind their respective target gene promoters at more proximal sites and then activate transcription. Unlike OKM, SOX2 remains enriched at distal enhancers.

rant overexpression can lead to transformation upon accumulation of other mutations in the context of tumorigenesis. More work needs to be performed to fully understand the mechanics and detailed temporal aspects of reprogramming.

CONCLUSIONS

MYC clearly plays an essential role in the processes that underpin early embryonic development and pluripotent stem cell biology. Functions in promoting transcriptional activation of genes responsible for self-renewal and those imposing a differentiation blockade have been well documented but exactly how these influence reprogramming at the molecular level remains an important problem. PSCs are likely to provide a critical biological platform for deciphering how MYC functions in a wide range of biological contexts.

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