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Letter

# Biallelic Expression of Nanog Protein in Mouse Embryonic Stem Cells

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Transcription factors (TFs) and their networks are central effectors controlling pluripotency (Young, 2011). Numerous involved TFs have been identified, but a subset of core pluripotency TFs regulates the majority of others. One such factor, Nanog, is expressed in pluripotent cells, is required for self-renewal of mouse embryonic stem cells (ESCs) in vitro, is able to force ESC self-renewal upon overexpression in the absence of LIF, and is necessary for the normal development of early mouse embryos (reviewed in Young, 2011). Several studies have shown that Nanog expression is heterogeneous in populations of pluripotent ESCs, which can express high or low Nanog levels (reviewed in Young, 2011), making Nanog regulation an interesting model for analyzing the dynamic regulation of fluctuating but stable TF expression states. Recently, allele-specific expression of *Nanog*—as assessed by a combination of fluorescent in situ hybridization (FISH) to detect *Nanog* mRNA and protein-based assays involving fusion of destabilized fluorescent proteins connected to Nanog via a self-cleavable peptide—has been described as a potential mechanism for regulation of *Nanog* expression and, consequently, pluripotency (Miyanari and Torres-Padilla, 2012). These studies suggested that Nanog is predominantly expressed in a monoallelic manner in serum/LIF-cultured ESCs but biallelically in 2i "ground state" conditions, and they led to the conclusion that switching to higher biallelic Nanog expression is associated with a more stable pluripotent state. However, the underlying mechanisms and functional relevance remained unclear.

To examine the allelic distribution of Nanog expression at the protein level, we created knockin ESC lines in which the two endogenous *Nanog* alleles are targeted with a yellow (VENUS) and red (KATUSHKA) fluorescent protein (FP), respectively (Figures S1A and S1B available online). The FPs are fused to the C terminus of the Nanog protein, so they reflect all of the regulatory mechanisms influencing the amount of Nanog protein in ESCs and measure functionally relevant levels of Nanog protein, not separate markers that could have different stability or regulation. To confirm the functionality of the Nanog-FP fusions, the pluripotency of the $Nanog^{VENUS/KATUSHKA}$ ESC reporter lines was tested in vitro and in vivo. Loss of *Nanog* leads to differentiation and loss of ESC maintenance, and Nanog-deficient embryos do not develop past the implantation stage (Mitsui et al., 2003). In contrast, $Nanog^{VENUS/KATUSHKA}$ ESCs survived and proliferated normally over at least 250 population doublings in vitro, exhibited normal morphology of undifferentiated ESCs (Figure S1C), and expressed other ESC-pluripotency-specific TFs like Oct3/4, Sox2 (Figure S1D), and Rex1 (data not shown). Both Nanog-FP reporters also showed normal downregulation during induced ESC differentiation upon LIF withdrawal (Figure S1H). We also verified the functionality of the $Nanog^{VENUS}$ and $Nanog^{KATUSHKA}$ fusion proteins through a tetraploid aggregation assay, the most stringent test for ESC pluripotency: normal day 9.5 embryos can be generated from $Nanog^{VENUS/KATUSHKA}$ ESCs without contribution of tetraploid cells (Figure S1E). In addition, the stability of $Nanog^{VENUS}$ and $Nanog^{KATUSHKA}$ fusion proteins is identical to that of wild-type Nanog protein (Figure S1F). Thus, the normal function and stability of $Nanog^{VENUS}$ and $Nanog^{KATUSHKA}$ fusion proteins indicates that they can be used as faithful reporters of Nanog protein expression.

We used the labeled cells to examine Nanog expression. As previously described (Chambers et al., 2007), we saw a range of Nanog expression levels when the ESCs were cultured in serum/LIF conditions, although the dynamic range was not as broad as in some previous reports. We found that the extent of this variability of Nanog expression depended on culture conditions and strain background and could also vary between genetically identical ESC clones. However, we unexpectedly did not see evidence for widespread monoallelic expression of Nanog protein (Figure S1G). Instead, Nanog expression was highly correlated between the two alleles in terms of the expression level within individual cells. This situation remained unchanged in ESCs cultured over many weeks (data not shown). Consistent with prior reports, Nanog expression changed to a more uniform high distribution in ESC populations cultured in 3i ground state conditions (Ying et al., 2008) (Figure S1G). We cannot exclude potential monoallelic Nanog protein expression in a very small subset (less than 2%) of ESCs due to potential noise levels of FACS analysis (individual dots in FACS plots of Figure S1G). We can, however, conclude that we do not see evidence for significant monoallelic Nanog expression in ESCs at the protein level. Although we did not analyze the potential for monoallelic Nanog protein expression in other ESC lines, the normal

self-renewal and pluripotency properties of our cells suggest that monoallelic regulation of expression is not required for wild-type Nanog function.

It is unclear at this point what the basis is for the difference between our results and those of Miyanari and Torres-Padilla (2012). One possible explanation could lie with transcriptional bursts, which seem to occur at a low frequency even for actively expressed genes (Suter et al., 2011). Thus, FISH data from one point in time might detect transcription of only one allele because of burst behavior rather than overall monoallelic *Nanog* expression. Differences in terms of stability between the separate reporter proteins and Nanog itself could also influence the results seen at the protein level.

It is important to note that we did not analyze the potential for allele-specific bias of *Nanog* transcription. However, even if it occurs, our data suggest that it would not lead to prevalence of Nanog protein from one allele in ESCs, and thus it is not likely to be functionally relevant as a central mechanism of regulating pluripotency or heterogeneity in pluripotency TF expression. Instead, we would suggest that other regulatory mechanisms, including Nanog autorepression (Fidalgo et al., 2012, Navarro et al., 2012) and the topology of the pluripotency TF and signaling networks (MacArthur et al., 2012), underlie the heterogeneous molecular states seen in individual pluripotent cells. A related paper in this issue from Faddah et al. (2013) draws similar conclusions to ours regarding biallelic expression of *Nanog*, and in addition looks more broadly at variability in *Nanog* expression at the transcriptional level and the activity of a range of reporter constructs. Together, these studies will help inform future analysis of the regulation of Nanog expression and pluripotency networks.

## SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures and one figure and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.04.025.

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