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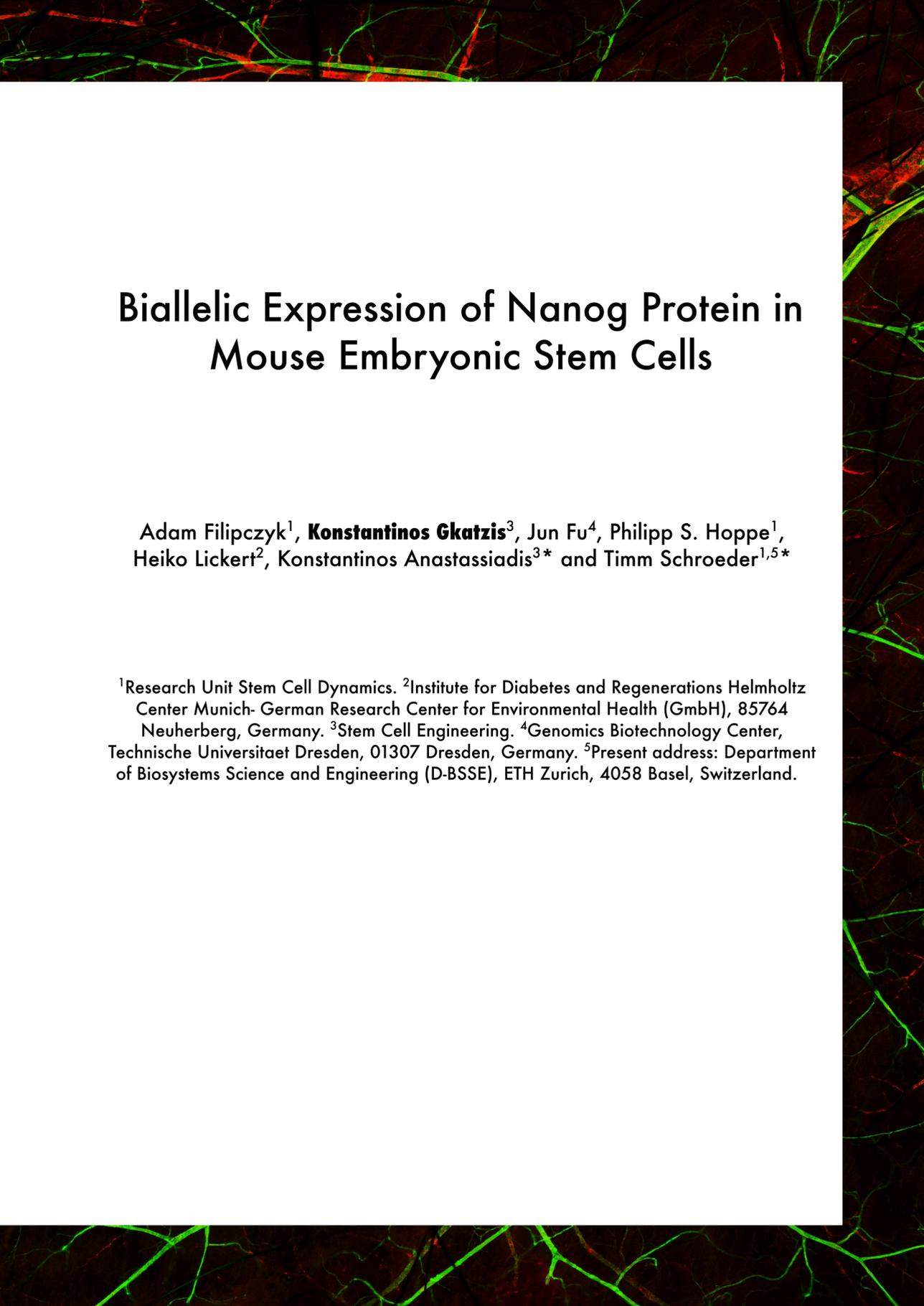
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Chapter

02

“...this knock-in fusion strategy allows the generation of functional Nanog fusion proteins mirroring normal protein distribution in vivo and in vitro...”



Biallelic Expression of Nanog Protein in Mouse Embryonic Stem Cells

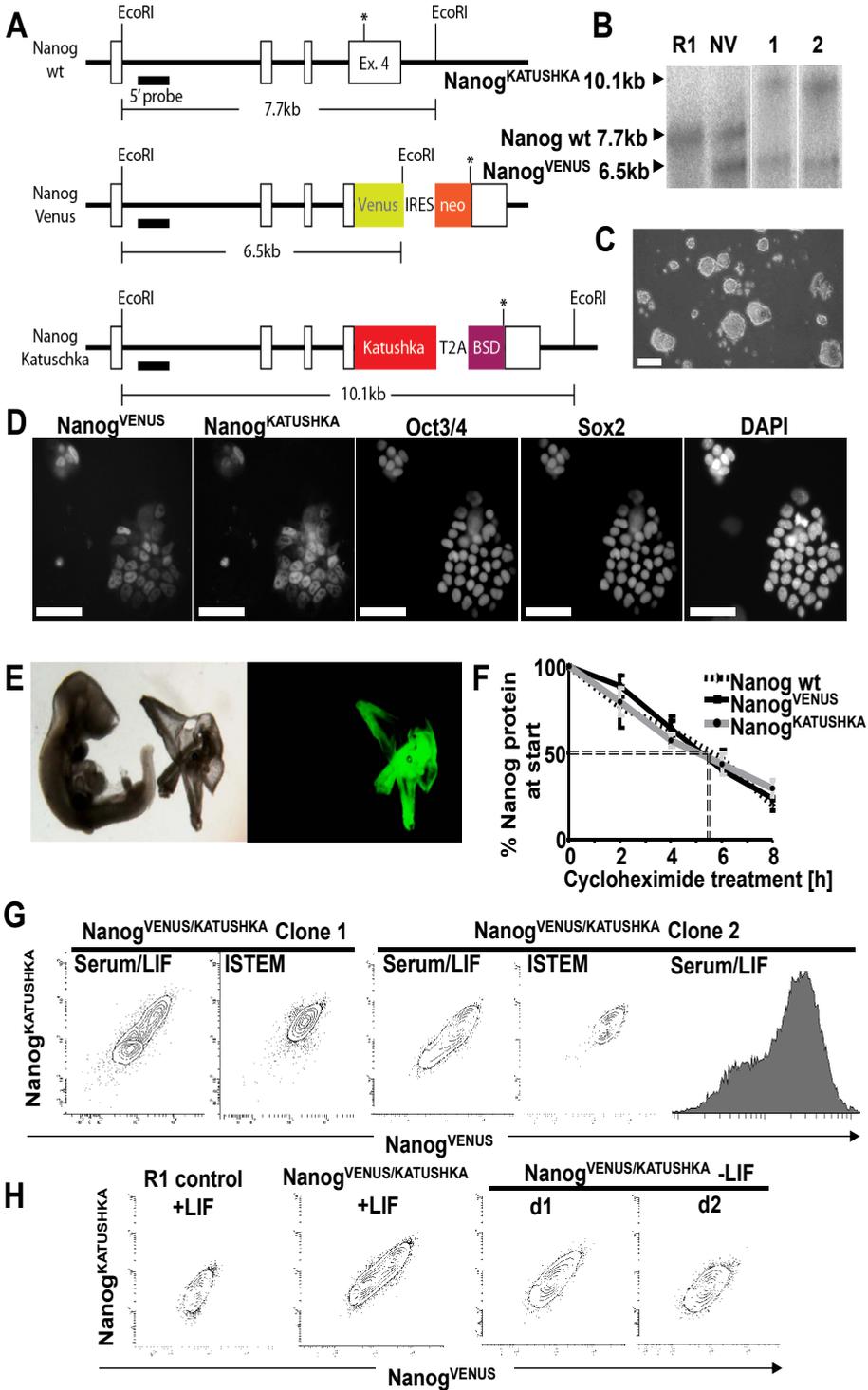
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Transcription factors (TFs) and their networks are central effectors controlling pluripotency (Young, 2011). Numerous involved TFs have been identified, but a subset of core pluripotency TFs regulates the majority of others. One such factor, *Nanog*, 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 (Young, 2011). Several studies have shown that *Nanog* expression is heterogeneous in populations of pluripotent ESCs, which can express high or low *Nanog* levels (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 (**Fig.1A** and **Fig.1B**). 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 (**Fig.1C**), and expressed other ESC-pluripotency-specific TFs like Oct3/4, Sox2 (**Fig.1D**), and Rex1 (data not shown).

Both *Nanog*-FP reporters also showed normal downregulation during induced ESC differentiation upon LIF withdrawal (**Fig.1H**). 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 (**Fig.1E**). In addition, the stability of *Nanog*^{VENUS} and *Nanog*^{KATUSHKA} fusion proteins is identical to that of wildtype *Nanog* protein (**Fig.1F**). 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 (**Fig.1G**). 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



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Figure 1: Nanog protein is expressed from both alleles in embryonic stem cells. A) Targeting strategy for generation of allele specific fluorescent Nanog protein reporter alleles. White boxes denote Nanog Exons; asterisk denotes Nanog stop codon in Exon 4. B) Southern Blot analysis showing correct targeting of Nanog^{VENUS/KATUSHKA} clones 1 and 2. Expected band sizes are 7.7kb for wt Nanog, 6.5kb for VENUS fusion, and 10.1kb for KATUSHKA fusion. Southern Blot bands shown were run on the same gel, lanes without correctly targeted clones were removed from panel to save space. R1: parental wild type R1 ESCs. NV: R1 ESCs with only one Nanog allele targeted with VENUS construct. C) and D) Normal functionality of Nanog^{VENUS} and Nanog^{KATUSHKA} protein fusions. C) Nanog^{VENUS/KATUSHKA} ESC clones maintain normal undifferentiated morphology (size bar: 200µm), and retain expression of other pluripotency transcription factors (size bar: 50µm) (D). E) Contribution of Nanog^{VENUS/KATUSHKA} ESCs to normal embryo development at 9.5dpc (left panel) as examined by tetraploid aggregation demonstrates normal function of Nanog-FP fusions. Tetraploid cells for the generation of extra embryonic tissues were derived from a mouse with ubiquitous eYFP expression (right panel). F) Normal stability of Nanog^{VENUS} and Nanog^{KATUSHKA} protein fusions. Unchanged protein degradation rates (upon Cycloheximide treatment) demonstrate normal stability of Nanog-FP fusions. G) Nanog protein is not expressed in an allele specific way in ESCs. ESC populations cultured in serum/LIF conditions exhibit the expected heterogeneous Nanog expression. As with other reporters, Nanog distributions which can vary between clones and cultures. Culture in serum free medium containing chemical inhibitors of differentiation (ISTEM) leads to homogeneous Nanog expression. H) Induction of ESC differentiation by LIF withdrawal leads to Nanog down regulation but does not induce its monoallelic expression.

prior reports, Nanog expression changed to a more uniform high distribution in ESC populations cultured in 3i ground state conditions (Ying et al., 2008) (**Fig.1G**). 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 **Fig.1G**). 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 (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. (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.

Methods

ESC culture. Mouse ESCs were cultured in DMEM basal medium (catalog number: 31053-044, Gibco, CA, USA) supplemented with 2mM L-Glutamine (catalog number: 25030-024, Gibco, CA, USA), 100U Penicillin/Streptomycin (catalog number: 15140-0122, Gibco, CA, USA), 1% Non-essential amino acids (catalog number: 11140-035, Gibco, CA, USA), 1mM Sodium Pyruvate (catalog number: S8636, Sigma, MO, USA), 10⁻⁴ M β-mercaptoethanol (catalog number: 31350-010, Gibco, CA, USA), 10% FCS (catalog number: 2602P250915, PAN, Aidenbach, Germany) and Leukemia Inhibitory Factor (LIF) on 0.1% porcine gelatin (Sigma, catalog number: G1890-100G) coated tissue culture grade plastic ware (Falcon, NJ, USA). ESCs were also cultured in serum free ISTEM medium (Ying et. al., 2008) (Catalog number: SCS-SF-ES-01, Stem Cells Inc. Cambridge, United Kingdom), or serum containing medium without LIF.

Generation of targeting constructs. All steps for the generation of the plasmids containing fluorescent reporters and the targeting constructs were done using recombineering technology. Details are available upon request.

ES Cell targeting. Mouse ESCs from the R1 parental line (129 sub-strain) were electroporated with linearized targeting vector and correct integration in Blasticidin (5 µg/ml, catalog number: A11139-02, Invitrogen, CA, USA) resistant ES clones was confirmed by Southern blotting. Nanog^{KATUSHKA} heterozygous fusion mESCs underwent a second round of electroporation and were selected by G418 (200 µg/ml, catalog number: G8168-10ML, Invitrogen, CA, USA) addition. Resistant mESCs were then FACS (Fluorescence Activated Cell Sorting) sorted for VENUS positivity, reselected in G418 and confirmed for correct integration by Southern blotting.

Imaging and Immunofluorescence Staining. ESCs were cultured on gelatin coated µ-slides (Catalog number: 80606, Ibbidi, Martinsried, Germany) in serum/LIF medium as described above. Images were acquired with a Zeiss Axio Observer Z1 microscope, an AxioCam Hrm camera (Zeiss, Munich, Germany) and a Lumencor Spectra-X multichannel light engine (Lumencor, Beaverton, USA). After cell fixation with 4% formaldehyde solution, it was confirmed that 100ms exposure (used for antibody detection below) does not yield detectable signals from fluorescent proteins. Cells were stained with a goat anti Oct3/4 antibody (1µg/ml, Catalog number: sc8628, Santa Cruz, USA) and a rabbit anti Sox2 (2.5µg/ml, Catalog number: Ab5603, Millipore, USA) primary antibody. Secondary antibodies donkey anti-goat IgG Alexa 488 (4µg/ml Catalog number: A-11055, Invitrogen, California, USA) and donkey anti-rabbit IgG Alexa 555 (4µg/ml Catalog number: A-31572 Invitrogen, California, USA), were then applied. Cells were counterstained with Roti-mount fluorcare DAPI solution and images of stained cells were re-acquired.

Fluorescence Activated Cell Sorting (FACS). Analysis of Nanog^{VENUS/KATUSHKA} double knockin ESCs was performed using a FACSAriaIII flow cytometer using a BD FACSDiva software package (Becton Dickinson, Heidelberg, Germany). The Blue 488nm laser combined with a 530/30 filter was used to detect Nanog^{VENUS} and the Yellow/Green 561nm laser combined with a 610/20 filter was used to detect Nanog^{KATUSHKA}. The R1 wt mESCs were used to set negative gates.

Generation of Chimeras. Tetraploid chimeras were generated according to standard protocols and aggregations with Nanog^{VENUS/KATUSHKA} double knockin ESCs were performed in YV1 (Yellow fluorescent protein Viral 1) (a kind gift from Andras Nagy and Marina

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Gerstenstein) and examined for contribution at embryonic day E10.5dpc.

Protein half-life assay. ES cultures were treated with 50 μ M Cycloheximide for 2, 4, 6 and 8 hour intervals after which cells were lysed in 0.1% NP40 lysis buffer. Lysates were resolved by western blotting on 10% polyacrylamide gels and Nanog protein was detected with rabbit anti-Nanog antibody ab80892 (Abcam, Cambridge, United Kingdom). Quantification of protein levels was performed by Image J software using the Gel analyzer feature to gate on protein lanes and to uniformly quantify band intensities over background (NIH, USA).

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