Title
T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer.

Permalink
https://escholarship.org/uc/item/05x372h0

Journal
The Journal of experimental medicine, 201(9)

ISSN
0022-1007

Authors
Cobb, Bradley S
Nesterova, Tatyana B
Thompson, Elizabeth
et al.

Publication Date
2005-05-01

DOI
10.1084/jem.20050572

Peer reviewed
T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer

Bradley S. Cobb, Tatyana B. Nesterova, Elizabeth Thompson, Arnulf Hertweck, Eric O’Connor, Jonathan Godwin, Christopher B. Wilson, Neil Brockdorff, Amanda G. Fisher, Stephen T. Smale, and Matthias Merkenschlager

1Lymphocyte Development Group, 2Developmental Epigenetics Group, 3Flow Cytometry Facility, and 4Transgenics Facility, Medical Research Council Clinical Sciences Centre, Imperial College London, London W12 0NN, England, UK
2Department of Immunology, University of Washington, Seattle, WA 98195
4Howard Hughes Medical Institute, Department of Microbiology, Immunology, and Molecular Genetics, and 5Molecular Biology Institute, University of California, Los Angeles, California 90095

The ribonuclease III enzyme Dicer is essential for the processing of micro-RNAs (miRNAs) and small interfering RNAs (siRNAs) from double-stranded RNA precursors. miRNAs and siRNAs regulate chromatin structure, gene transcription, mRNA stability, and translation in a wide range of organisms. To provide a model system to explore the role of Dicer-generated RNAs in the differentiation of mammalian cells in vivo, we have generated a conditional Dicer allele. Deletion of Dicer at an early stage of T cell development compromised the survival of αβ lineage cells, whereas the numbers of γδ-expressing thymocytes were not affected. In developing thymocytes, Dicer was not required for the maintenance of transcriptional silencing at pericentromeric satellite sequences (constitutive heterochromatin), the maintenance of DNA methylation and X chromosome inactivation in female cells (facultative heterochromatin), and the stable shutdown of a developmentally regulated gene (developmentally regulated gene silencing). Most remarkably, given that one third of mammalian mRNAs are putative miRNA targets, Dicer seems to be dispensable for CD4/8 lineage commitment, a process in which epigenetic regulation of lineage choice has been well documented. Thus, although Dicer seems to be critical for the development of the early embryo, it may have limited impact on the implementation of some lineage-specific gene expression programs.

Small RNA molecules have important functions in gene regulation, chromatin structure, and chromosome maintenance in a wide range of organisms (1–7). The RNase III enzyme Dicer is required for the processing of short (21–22 nucleotides) micro-RNAs (miRNAs) and small interfering RNAs (siRNAs) from double-stranded RNA precursors. Dicer-generated RNAs trigger the destruction of complementary miRNAs or prevent their translation, and may recruit chromatin modifiers to sites of repetitive DNA sequences or to specific promoters (1–7). Each of several hundred miRNA genes may regulate multiple transcripts, so that one in three protein-coding transcripts could be subject to miRNA regulation (8, 9).

Defining the role of Dicer-generated RNAs in mammalian development is complicated by embryonic lethality of constitutive Dicer knockouts in mice (10, 11). Mouse embryonic stem cells that are selected for viability in the absence of Dicer fail to differentiate in vitro and do not contribute to mouse development in vivo (6); this may point to a role for siRNAs and miRNAs in the regulation of gene expression or differentiation.

An involvement of miRNAs in hematopoiesis is suggested by the position of miRNA genes near translocation breakpoints or deletions in human leukemias (12–14). Several miRNAs are restricted to hematopoietic cells and the enforced expression of miR–181 in progenitor cells favors the development of B over T cells; this indicates that miRNAs may contribute to the control of hematopoiesis (15).

Lymphocytes may be of use to investigate Dicer functions, because in contrast with cell lines and early embryos, lymphocytes spend ex-
tended periods in a resting state. Moreover, their differentiation is well-studied. Early T cell precursors, double negative (DN) for CD4 and CD8, proliferate while they progress through the CD44+CD25+ (DN1) and the CD44+CD25- (DN2) stages to the CD44-CD25+ (DN3) stage. Precursors of the TCR-γδ lineage diverge at the DN stage (16). DN3 cells that are committed to the TCRαβ lineage remain in a nonproliferating (G1) state until productive TCR-β rearrangement occurs and preTCR signals trigger reentry into the cell cycle, loss of CD25 (DN4), and the acquisition of CD4 and CD8. Cell division that is driven by the preTCR stops soon after thymocytes become CD4 CD8 double positive (DP), and subsequent differentiation occurs without obligatory proliferation (16). DP thymocytes are bipotential progenitors of CD4+ helper and CD8+ cytotoxic T cells. In response to TCR engagement, DP thymocytes elevate the expression of the activation markers, CD5 and CD69; transiently down-regulate the lineage markers, CD4 and CD8; and silence genes that are involved in TCR rearrangement, including Rag and Tdt. They initiate lineage-specific gene expression programs and differentiate via a series of intermediates (DPlo, CD4lo8Hi; CD4Hi8lo) into CD4 or CD8 single positive (SP) thymocytes (16). We have constructed a conditional allele and used lineage-specific Cre expression to delete Dicer during T cell development in the thymus.

RESULTS AND DISCUSSION

Dicer deletion in thymocytes

To explore the role of Dicer in T lymphocyte development, we flanked an essential RNaseIII domain (exons 20 and 21) with loxP sites to create Dicerloxp (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050572/DC1). Mice that were homozygous for this allele were viable, fertile, and had no obvious defects in lymphocyte development. When we introduced an lckCre transgene—which is active from the DN3 stage requires the productive rearrangement and expression of TCR and TCRβ (16). Expression of TCRβ was not compromised by Dicer deletion, and intracellular staining showed the expected increase in TCRβ expression between the small and the large DN3 stage (Fig. 2 c). Correspondingly, analysis of DNA content showed similar proportions of actively cycling Dicerloxp/lox and lckCre DicerAΔ DN thymocytes (Fig. 2 d).

An unusually high percentage of lckCre DicerAΔ thymocytes expressed TCR-γδ (6.7 ± 2.7%, n = 4) compared with 0.4 ± 0.2% in Dicerloxp/lox (n = 3), and γδ cells were prevalent in the DN compartment (Fig. 2 e). As in DP thymocytes, Dicer deletion was virtually complete in lckCre DicerAΔ γδ cells (Fig. 2 f); however, in contrast to αβ cells, γδ cell numbers were reduced in lckCre DicerAΔ thymi (7.3 ± 1 × 10⁵ per lckCre DicerAΔ thymi, n = 4; 5.7 ± 3 × 10⁵ per Dicerloxp/lox thymi, n = 3). This abundance of γδ cells might be explained, paradoxically, by the limited expansion of γδ relative to preTCR-expressing αβ precursor (16). Fewer cell divisions could mean preferential survival in the absence of Dicer. Alternatively, Dicer-dependent mechanisms may control αβ/γδ lineage choice directly. Deficient Notch/RBP-J signaling favors γδ relative to αβ cells (18, 19) and Notch signaling components are among predicted miRNA targets (9).
BRIEF DEFINITIVE REPORT

Increased susceptibility to cell death

Because there was no indication for a developmental block at the DN stage, we looked at cell death as an alternative explanation for the reduced numbers of lckCre Dicer^{ΔΔ} thy-mocytes. Ex vivo, few thymocytes stained with Annexin V (unpublished data) or showed reduced mitochondrial membrane potential as an early marker of apoptosis (20). In vitro culture revealed more dying lckCre Dicer^{ΔΔ} thymocytes than controls (43% versus 11% at 6 h and 68% versus 30% after 24 h; Fig. 3).

Dicer deficiency has been linked to heterochromatin defects (1, 5, 6) and centromere dysfunction in dividing cells (1, 5), which might result in checkpoint activation and/or missegregation of genetic material (1, 5). The generation of DP cells from the DN1/2 stage involves six to eight divisions (16). In contrast to lckCre Dicer^{ΔΔ} mice, CD4Cre Dicer^{ΔΔ} mice (where Cre is expressed slightly later; reference 17) have relatively normal thymocyte numbers (unpublished data); this suggests that the time or the number of cell divisions between the deletion of Dicer and the DP stage may affect thymocyte survival. Alternatively, Dicer-dependent RNAs might regulate survival directly (21).

Maintenance of constitutive and facultative heterochromatin

We used RT-PCR to evaluate heterochromatic silencing. Major and minor satellite transcripts were readily detectable in lckCre Dicer^{ΔΔ} thymocytes. (c) Intracellular staining of DN thymocyte subsets indicates normal TCR-β expression in lckCre Dicer^{ΔΔ} DN thy-mocytes. (d) DNA content as assessed by propidium iodide (PI) staining indicates that lckCre Dicer^{ΔΔ} DN thymocytes proliferate normally. (e) TCR γδ-expressing cells are overrepresented in the absence of Dicer. (f) Genomic PCR shows that Dicer deletion is comparable, and virtually complete, in lckCre Dicer^{ΔΔ} γδ-expressing thymocytes.

Figure 3. Increased cell death in the absence of Dicer. Thymocytes were stained for CD4, CD8, and DiOC6 as an indicator of mitochondrial membrane potential ex vivo or after culture. Histograms are gated on DP cells but not on light scatter.
in differentiating muscle cells, but not in control or Dicer-deficient thymocytes (Fig. 4 a).

The genome is subject to silencing during progressive lineage restriction (22) and the silent X chromosome in female cells provides a tractable model for facultative heterochromatin (23). We used RNA fluorescence in situ hybridization (FISH) to determine if the expression and localization of the noncoding RNA Xist, which is required for X inactivation (23), are affected in Dicer-deficient cells. In control XX somatic cells, Xist RNA highlights the territory of the inactive X chromosome in control Dicerlox/lox and lckCre Dicer^{lox/loxCre} Dicer^{lox/loxAlox} DP thymocytes (Fig. 4 b and Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20050572/DC1). We also assessed if there was reactivation of the X-linked Pgk-1 gene, which would result in the appearance of two foci per cell, or Pgk-1 foci within Xist domains. Although RNA FISH may not detect very low levels of expression, the results rule out substantial Pgk-1 reactivation in lckCre Dicer^{lox/loxCre} Dicer^{lox/loxAlox} DP cells (n = 170) relative to controls (n = 135; Fig. 4 b).

Multiple, partially redundant mechanisms maintain X inactivation; disruption of only one of these may not be sufficient for X reactivation. Based on recent data that siRNAs can direct deoxycytosine-deoxyguanosine (CpG) island methylation (3, 4), we were interested in DNA methylation of X-linked CpG islands, which normally are unmethylated on active X chromosomes and fully methylated on the inactive X (unpublished data). Using methylation-sensitive restriction enzymes to examine MeCp2 and Mtm1 CpG islands, we observed approximately equal levels of uncut (methylated) and cut (unmethylated) bands which corresponded to alleles on the active and the inactive X, respectively, in female control Dicerlox/loxCre Dicer^{lox/loxAlox} Dicer^{lox/loxAlox} thymocyte DNA (Fig. 4 c). Hence, at this level of analysis, the maintenance of constitutive and facultative heterochromatin seemed to be unperturbed in lckCre Dicer^{loxAlox} Dicer^{lox/loxAlox} thymocytes.

**CD4/CD8 lineage choice and differentiation**

Given the role of siRNAs and miRNAs in the regulation of gene expression and differentiation in other systems (1–7), it was of interest to determine how the loss of Dicer at the DN stage would affect the sequence of events during the transition from the DP to the SP stage of thymocyte development. Despite the reduced cellularity of the DP thymocyte compartment, the frequency of CD5hi and CD69hi cells was similar to controls; this indicates that a normal proportion of DP thymocytes was recruited into the thymic selection process (Fig. 5 a and reference 24). DPlo and CD48lo cells in transit to the SP populations and CD4 and CD8 SP cells were present at the expected frequencies (Figs. 2 a and 5 b). As part of their intrathymic maturation, CD4SP cells gradually down-regulate CD69 and CD24 (HSA; reference 16); this was not perturbed in lckCre Dicer^{loxAlox} Dicer^{lox/loxAlox} thymocytes (Fig. 5 c).
In addition to the mutually exclusive expression of CD4 and CD8, mature thymocyte subsets differentially express "signature" genes, such as Ph-POK in the CD4 lineage and perforin and cathepsin W in the CD8 lineage (25, 26). Quantitative RT-PCR (Fig. 5 d) confirmed the appropriate expression of Ph-POK in CD4 but not in DP or CD8 SP lckCre Dicer^+/H9004/H9004 thymocytes (25). Perforin and cathepsin W were expressed more highly in CD8 SP than in DP or CD4 SP thymocytes (26).

Developmentally regulated gene silencing

Owing to TCR specificity and other constraints, only a relatively small proportion of DP thymocytes differentiate from the DP to SP stage, even in wild-type mice (16, 24). To address whether the entire population of lckCre Dicer^+/H9004/H9004 thymocytes is able to undergo early differentiation events, we used an in vitro differentiation model in which DP thymocytes that are exposed to surrogate TCR signals (phorbol ester and calcium ionophore) silence Tdt expression (27, 28). The great majority of control Dicer^lox/lox and lckCre Dicer^+/H9004/H9004 DP cells up-regulated CD5 and CD69 (not depicted); Tdt RNA expression declined to levels that were comparable with Dicer^lox/lox controls (Fig. 5 e). This indicates that most, if not all, lckCre Dicer^+/H9004/H9004 DP cells were competent to down-regulate Tdt.

Initially, Tdt silencing is reversible, so that Tdt is reexpressed when TCR stimulation ceases (28). Only after several hours of continued signaling does Tdt silencing become a stable trait, which in normal thymocytes—but not in certain thymoma cell lines—persists even after removal of the stimulus (28). To address whether lckCre Dicer^+/H9004/H9004 DP thymocytes silence Tdt in a stable fashion, we initiated silencing by culture with phorbol ester and calcium ionophore, removed the stimulus, and recultured the cells for 10 h. Neither Dicer^lox/lox nor lckCre Dicer^+/H9004/H9004 DP cells reexpressed Tdt; this indicates that Dicer-deficient cells are competent to establish stable gene silencing (Fig. 5 e). Developmentally regulated silencing of Tdt during the in vivo differentiation of DP thymocytes also was intact, because lckCre Dicer^+/H9004/H9004 CD4 SP thymocytes showed equivalent levels of Tdt down-regulation ex vivo (Fig. 5 e).

Conclusions

Our analysis reveals a requirement for Dicer in the generation and survival of normal numbers of αβ T cells. In contrast, Dicer apparently is not essential for the maintenance of transcriptional silencing of pericentromeric satellite sequences (constitutive heterochromatin), the maintenance of X chromosome inactivation and cytosine methylation in female
cells (facultative heterochromatin), or the stable shutdown of a developmental stage-specific gene (developmentally regulated gene silencing) in the T cell lineage. These results do not question the general involvement of Dicer in the maintenance of heterochromatin (1, 5), but suggest that Dicer may not be required continually for heterochromatin maintenance in thymocytes. We have not investigated centromere structure and function directly, but our RT-PCR analysis of major and minor satellite transcripts gives no indication of transcriptional derepression. It is likely that epigenetic marks, such as CpG methylation—once established during development—allow for Dicer-independent maintenance of heterochromatin. Current estimates suggest that as many as one in three mRNAs are targets of miRNA regulation (9). Given the important roles that are ascribed to small, double-stranded RNAs in the regulation of gene expression and differentiation (1–7), it is remarkable that Dicer appears to be dispensable for CD4/8 lineage commitment and the implementation of lineage-specific gene expression programs.

MATERIALS AND METHODS

Construction of Dicer targeting vector. Details of the targeting vector are shown in Fig. S1. The vector was electroporated into ES cells and homologous recombination was assayed by the Southern strategy that is outlined in Fig. 1. One of several correctly targeted ES cell clones (clone 96.2) was used for the production of chimeric mice by blastocyst injection.

Mouse strains, cell sorting, and culture. Animal work was performed according the Animals (Scientific Procedures) Act, UK. Dicerlox/lox mice were crossed with LckCre transgenic mice (17) to generate lckCrelox/lox mice. Thymocytes were stained, analyzed, and sorted by flow cytometry as described previously (24). Where indicated, thymocytes were incubated with 40 nM DiOC6 (Molecular Probes) for 10 min at 37°C as described previously (20). To down-regulate Tdt expression, DP thymocytes were cultured with 7.5 ng/ml PMA (Sigma–Aldrich) and 180 ng/ml ionomycin (Sigma–Aldrich) as described previously (28).

RNA FISH. RNA FISH for Xist and Pgk was done as described previously (29) with minor modifications. FACSort-sorted thymocytes were prefixed in 1% paraformaldehyde for 10 min on ice, 100 μl of cell suspension (~6 × 10^6) was cytospun onto glass slides, permeabilized with 0.5% Triton in ice-cold cytoskeletal buffer for 5 min, and postfixed with 4% paraformaldehyde for 10 min on ice. Slides were stored in 70% ethanol.

CpG methylation analysis. DNA from control XY cells, XX Dicerlox/lox, and XX lckCre Dicerlox/lox thymocytes was digested with XbaI and NruI (MseCI) or XbaI and MluI (MnlI), electrophoresed on 1% agarose gels, Southern blotted, and hybridized using standard procedures.

RT-PCR. Total RNA was isolated using RNAbee (Tel-Test) and reverse transcribed. Real-time PCR analysis was performed on a Opticon DNA Engine (MJ Research Inc.) and normalized as described previously (30). Primer sequences and PCR conditions are available on request.

Online supplemental material. Figs. S1 and S2 describe the construction of the targeted Dicer allele and depict RNA FISH data on Xist expression. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050572/DC1.

We thank Drs. C.-Z. Chen for advice on miRNA northern, R. Terranova for cDNAs and help with the detection of centromeric transcripts, and Z. Webster and J. Mardon-Srivastava for help with ES cells and cell sorting.

This research was supported by the Medical Research Council, UK, and the National Institutes of Health, USA. The authors have no conflicting financial interests.

Submitted: 17 March 2005
Accepted: 29 March 2005

REFERENCES

1. Volpe, T.A., C. Kidner, I.M. Hall, G. Teng, S.I. Grewal, and R.A. Martienssen. 2002. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science. 297:1833–1837.

2. Bartel, D.P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 116:281–297.

3. Kawasaki, H., and K. Taira. 2004. Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. Nature. 431: 211–217.

4. Morris, K.V., S.W. Chan, S.E. Jacobsen, and D.J. Looney. 2004. Small interfering RNA-induced transcriptional gene silencing in human cells. Science. 305:1289–1292.

5. Fukagawa, T., M. Nomori, M. Yoshikawa, M. Ikeno, T. Okazaki, Y. Takami, T. Nakayama, and M. Oshimura. 2004. Dicer is essential for formation of the heterochromatin structure in vertebrate cells. Nat. Cell Biol. 6:784–791.

6. Kanellopoulou, C., S.A. Muljo, A.L. Kung, S. Ganesan, R. Drapkin, T. Jenuwein, D.M. Livingston, and K. Rajewsky. 2005. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. Genes Dev. 19:489–501.

7. Kuvabara, T., J. Hsieh, K. Nakashima, K. Taira, and F.H. Gage. 2004. A small modulatory dsRNA specifies the fate of adult neural stem cells. Cell. 116:777–793.

8. Lewin, B.P., C.B. Burge, and D.P. Bartel. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 120:15–20.

9. Lim, L.P., N.C. Lau, P. Garrett–Engele, A. Grimson, J.M. Schelter, J. Castle, D.P. Bartel, P.S. Lim, and J.M. Johnson. 2005. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature. 433:769–773.

10. Bernstein, E., S.Y. Kim, M.A. Carmell, E.P. Murchison, H. Alcorn, M.Z. Li, A.A. Mills, S.J. Elledge, K.V. Anderson, and G.J. Hannon. 2003. Dicer is essential for mouse development. Nat. Genet. 35:215–217.

11. Yang, W.J., D. Yang, S. Na, G. Sandusky, Q. Zhang, and G. Zhao. 2004. Dicer is required for embryonic angiogenesis during mouse development. J. Biol. Chem. 280:9330–9335.

12. Calm, G.A., C.D. Dumitru, M. Shimizu, R. Bichi, S. Zupo, E. Noch, H. Aiderl, S. Rattan, M. Keating, K. Ryu, et al. 2002. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc. Natl. Acad. Sci. USA. 100:1524–1529.

13. Gauwerky, C.E., K. Haeuber, M. Isebo, P.C. Nowell, and C.M. Croce. 1989. Activation of MYC in a masked (8;17) translocation results in an aggressive B-cell leukemia. Proc. Natl. Acad. Sci. USA. 86:8867–8871.

14. Lagos-Quintana, M., R. Rauhut, W. Lendeckel, and T. Tuschl. 2001. Identification of novel genes encoding for small expressed RNAs. Science. 294:853–858.

15. Chen, C.Z., L. Li, H.F. Lodish, and D.P. Bartel. 2004. MicroRNAs modulate hematopoietic lineage differentiation. Science. 303:83–86.

16. Kiselew, P., and H. von Boehmer. 1995. Development and selection of T cells: facts and puzzles. Adv. Immunol. 58:87–209.

17. Lee, P.P., D.R. Fitzpatrick, C. Beard, H.K. Jessup, S. Lehar, K.W. Makar, M. Perez-Melgosa, M.T. Sweetser, M.S. Schlüssel, S. Nguyen, et al. 2001. A critical role for Dmrt1 and DNA methylation in T cell development, function, and survival. Immunity. 15:763–774.

18. Tanigaki, K., M. Takeuchi, Y. Takada, N. Inoue, M. Kubo, and T. Honjo. 2004. Regulation of miR-17/92 T cell lineage commitment and peripheral T cell responses by Notch1/RBP-J signaling. Immunity. 20:611–622.

19. Washburn, T., E. Schweghoff, T. Gridley, D. Chang, B.J. Fowlkes, and A.L.
D. Cado, and E. Robey. 1997. Notch activity influences the alpha-beta versus gamma-delta T cell lineage decision. Cell. 88:833–843.

20. Zamzami, N., P. Marchetti, M. Castedo, D. Decaudin, A. Macho, T. Hirsch, S.A. Susin, P.X. Petit, B. Mignotte, and G. Kroemer. 1995. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J. Exp. Med. 182:367–377.

21. Xu, P., M. Guo, and B.A. Hay. 2004. MicroRNAs and the regulation of cell death. Trends Genet. 20:617–624.

22. Fisher, A.G. 2002. Cellular identity and lineage choice. Nat. Rev. Immunol. 2:977–982.

23. Brockdorff, N. 1998. The role of Xist in X-inactivation. Curr. Opin. Genet. Dev. 8:328–333.

24. Merkenschlager, M., D. Graf, M. Lovatt, U. Bommhardt, R. Zamoyska, and A.G. Fisher. 1997. How many thymocytes audition for selection? J. Exp. Med. 186:1149–1158.

25. He, X., X. He, V.P. Dave, Y. Zhang, X. Hua, E. Nicolas, W. Xu, B.A. Roe, and D.J. Kappes. 2005. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. Nature. 433:826–833.

26. Liu, X., and R. Bosselut. 2004. Duration of TCR signaling controls CD4-CD8 lineage differentiation in vivo. Nat. Immunol. 5:280–288.

27. Brown, K.E., J. Baxter, D. Graf, M. Merkenschlager, and A.G. Fisher. 1999. Dynamic repositioning of genes in the nucleus of lymphocytes preparing for cell division. Mol. Cell. 3:207–217.

28. Su, R.-C., K.E. Brown, S. Saaber, A.G. Fisher, M. Merkenschlager, and S.T. Smale. 2004. Assembly of silent chromatin at a developmentally regulated gene. Nat. Genet. 36:502–506.

29. Heard, E., F. Mongelard, D. Arnaud, and P. Avner. 1999. Xist yeast artificial chromosome transgenes function as X-inactivation centers only in multicopy arrays and not as single copies. Mol. Cell. Biol. 19:3156–3166.

30. Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalisation of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3:7. Epub 2002 Jun 18.