Targeting of \( >1.5 \) Mb of Human DNA into the Mouse X Chromosome Reveals Presence of \textit{cis}-Acting Regulators of Epigenetic Silencing

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ABSTRACT Regulatory sequences can influence the expression of flanking genes over long distances, and X chromosome inactivation is a classic example of \textit{cis}-acting epigenetic gene regulation. Knock-ins directed to the \textit{Mus musculus} \textit{Hprt} locus offer a unique opportunity to analyze the spread of silencing into different human DNA sequences in the identical genomic environment. X chromosome inactivation of four knock-in constructs, including bacterial artificial chromosome (BAC) integrations of over 195 kb, was demonstrated by both the lack of expression from the inactive X chromosome in females with nonrandom X chromosome inactivation and promoter DNA methylation of the human transgene in females. We further utilized promoter DNA methylation to assess the inactivation status of 74 human reporter constructs comprising \( >1.5 \) Mb of DNA. Of the 47 genes examined, only the \textit{PHB} gene showed female DNA hypomethylation approaching the level seen in males, and escape from X chromosome inactivation was verified by demonstration of expression from the inactive X chromosome. Integration of \textit{PHB} resulted in lower DNA methylation of the flanking \textit{HPRT} promoter in females, suggesting the action of a dominant \textit{cis}-acting escape element. Female-specific DNA hypermethylation of CpG islands not associated with promoters implies a widespread imposition of DNA methylation during X chromosome inactivation; yet transgenes demonstrated differential capacities to accumulate DNA methylation when integrated into the identical location on the inactive X chromosome, suggesting additional \textit{cis}-acting sequence effects. As only one of the human transgenes analyzed escaped X chromosome inactivation, we conclude that elements permitting ongoing expression from the inactive X are rare in the human genome.

\textit{X} chromosome inactivation (XCI) transcriptionally silences an X chromosome early in mammalian development, thereby serving as a means of dosage compensation between the sexes (reviewed in Migeon 2011). XCI is a classic paradigm of epigenetic silencing, but while many of the molecular players in the inactivation process have been elucidated (Wutz 2011), less is known about the \textit{cis}-acting DNA elements involved in the spread of silencing. A substantial number of X-linked genes continue to be expressed from the inactive X (Xi) in humans, and the clustered nature of these escapees in humans suggests that escape from XCI may be regulated in domains (Carrel and Willard 2005). Intriguingly, the number of escapees in mouse is considerably more limited, with only \( \sim 4\% \) of genes escaping XCI (~20 transcripts) in mice compared to \( \sim 15\% \) (~94 transcripts) of analyzed human genes (Carrel and Willard 2005; Lopes \textit{et al.} 2010; Yang \textit{et al.} 2010; Splinter \textit{et al.} 2011). The ongoing escape from inactivation of the escapee \textit{Kdm5c} when integrated at four different locations on the mouse X chromosome, while its flanking genes maintain their inactive state, provides strong evidence for intrinsic regulatory sequences, which we refer to as escape elements, that allow genes to escape from XCI (Li and Carrel 2008).

Gartler and Riggs (1983) proposed the existence of waystations or booster elements that promote the spread of inactivation, based on the limited inactivation of autosomal regions of X;autosome translocations, and Lyon proposed
that long interspersed elements (LINEs) that are enriched on the X chromosome could be such waystations (Waterston et al. 2002; Ross et al. 2005; Lyon 2006). Computational studies comparing the genomic neighborhoods of genes with different inactivation status have supported that inactivated and escape genes have different genomic environments with respect to the content of repetitive sequences (Bailey et al. 2000; Carrel et al. 2006; Wang et al. 2006; Nguyen et al. 2011). Several mouse escapees also escape from XCI in humans, and the difference in inactivation of flanking genes has been attributed to the presence or loss of the CTCF boundary element (Filippova et al. 2005; Goto and Kimura 2009). A direct comparison of the inactivation status of sequences integrated into the same genomic location would permit a direct analysis of the impact of cis-acting sequences on XCI and potentially identify sequences containing escape elements, waystations, and/or boundary elements.

Most transgenes on the X chromosome are subject to XCI with the only transgenes shown to consistently escape XCI being a chicken transferrin transgene and the mouse Kdm5c bacterial artificial chromosome (BAC) transgene (Goldman et al. 1987, 1998; Li and Carrel 2008), although a human collagen and an NF-kB-dependent EGFP reporter gene may also at least partially escape from XCI (Wu et al. 1992; Magness et al. 2004). Lack of knowledge of the integration site for the chicken transferrin transgene confounds the identification of cis-acting regulatory elements. Targeted single-copy integrations into a single location would provide a consistent resource to examine the spread of XCI into sequences not normally X linked, and Hprt has been described as a permissive locus with relatively minimal effect on transgene expression (Bronson et al. 1996; Cvetkovic et al. 2000). To assess whether a gene is subject to XCI many studies have examined allelic expression in females with nonrandom inactivation (e.g., Carrel and Willard 2005; Yang et al. 2010); however, an indirect means of assessing XCI is to study DNA methylation, which can be readily examined on banked DNA samples. CpG island promoters of X-linked genes that are subject to XCI on the Xi are typically DNA hypermethylated, while CpG island promoters are unmethylated on the active X (Xa), as on the autosomes (Wu et al. 1992; Chong et al. 2002; Matarasso et al. 2002; Cotton et al. 2009; Yasukochi et al. 2010). Genes and transgenes that escape XCI demonstrate low levels of promoter DNA methylation on both the Xa and the Xi (Goodfellow et al. 1988; Goldman et al. 1998), resulting in overall low DNA methylation levels in both males and females, allowing DNA methylation to be used to assess XCI status of genes (Cotton et al. 2009; Yasukochi et al. 2010).

Integration of transgenes at a single locus on the X chromosome allows for detailed examination of cis-acting DNA elements and we herein report the analysis of 74 human autosomal and X-linked transgenes knocked into the mouse Hprt locus that were generated from the Pleiades Promoter Project (Yang et al. 2009; Portales-Casamar et al. 2010; J.-F. Schmouth and E. M. Simpson, unpublished data), including eight human autosomal BACs of >195 kb integrated using the HuGx method [high-throughput human genes on the X chromosome (Schmouth et al. 2012)]. Overall there was a significant difference in DNA methylation between males and females, and most human promoters integrated into the X chromosome showed DNA hypermethylation in females, suggesting they were subject to XCI. Silencing from the Xi was verified by expression analysis of four BAC-derived transgenes present on the Xi in females that had nonrandom XCI. We identified one transgene that escaped from XCI and demonstrated that different constructs had differential capacity for DNA methylation when on the Xi, suggesting the presence of additional cis-acting epigenetic modulators.

Materials and Methods

Pleiades Promoter Project constructs

The Pleiades Promoter Project (Yang et al. 2009; Portales-Casamar et al. 2010) was an international collaborative effort to develop various human promoters driving specific expression patterns in the mouse brain, eye, and spinal cord. Most of the promoters originated from human autosomal genes, with only two X-linked promoters, DCX and MAOA, being assessed. All Pleiades strains were made using homologous recombination at the Hprt<sup>b-m3</sup> deletion locus on the mouse X chromosome; integration of the Pleiades constructs generated a chimeric Hprt<sup>b-m3</sup>/Hprt locus containing the human HPRT promoter that rescued the deletion (Bronson et al. 1996). MiniPromoters (MiniPs) were ≤4 kb in size and were composed of different combinations of small putative regulatory elements (http://pleiades.org/). In contrast, MaxiPromoters (MaxiPs) were human BAC-derived constructs that ranged from 100 to 195 kb of human genomic DNA, with a reporter inserted at the start codon of the gene of interest (J.-F. Schmouth and E. M. Simpson, unpublished data). For the MiniP constructs, the reporter lacZ or EGFP (or EGFP/cre) is ~200 bp or 50 bp downstream of the MiniP, respectively. Thirty-seven of 57 target genes contained a promoter CpG island, which was generally truncated in MiniPs compared to the endogenous islands (Supporting Information, Table S1). Approval for the generation and breeding of mice carrying the Pleiades constructs was obtained from the University of British Columbia Committee on Animal Care.

Generation of mouse strains

The floxed Xist strain 129-Xist<sup>fl<sup>lox</sup></sup> [stock no. 029172-UNC (Csankovszki et al. 1999)] was obtained heterozygous from the Mutant Mouse Regional Resource Center, and the cre-deleter strain FVB/N-Tg(CTCB-cre)2Mrt [stock no. 003376 (Lewandoski et al. 1997)] was obtained heterozygous from The Jackson Laboratory (JAX). Both strains were maintained by backcrossing to strain 129S1/SvImJ [stock no. 002448 (Simpson et al. 1997)] obtained from JAX. The floxed Xist strain was crossed to the cre-deleter strain at backcross generations JAX-plus N2 and N7, respectively, to
generate females carrying the Xist deletion (129-XXist\textsuperscript{lox}/X). Females with the Xist deletion were then crossed to males with the Pleiades construct integrated at the Hprt locus (B6-X\textsuperscript{MaxiP}/Y) to generate 129B6F1-XXist\textsuperscript{lox}/X\textsuperscript{MaxiP} females. This Xist knockout has been shown to render the X chromosome carrying it unable to inactivate (Gribnau et al. 2005), thus resulting in the MaxiP knock-in X chromosome with an intact Xist becoming the Xi. Complete nonrandom XCI was verified by examining the relative expression levels of the B6 and 129 alleles at a single-nucleotide polymorphism of the Fln locus (data not shown).

The B6.129S4-Gt(Rosa)26Sor\textsuperscript{em1Sor}/J strain [stock no. 003474 (Soriano 1999)] was obtained homozygous from JAX at N5, maintained by backcrossing to strain C57BL/6J (stock no. 000664) obtained from JAX, and then made homozygous again at N10. The allele was introduced into the Pleiades strains at N5–N10 (Portales-Casamar et al. 2010).

DNA and RNA extraction from tissues

An ear notch of \~1 mm in diameter was taken from each mouse postweaning (~4 weeks old) and digested with 200 µl of mouse homogenization buffer [50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM MgCl\textsubscript{2}, 0.1 mg/ml gelatin, 0.45% IGEPAL CA-630 (Sigma-Aldrich), 0.45% Tween 20 (Sigma-Aldrich), and 24 µg of Proteinase K (Sigma-Aldrich)] overnight at 55\textdegree. The digested samples were then heat inactivated at 95\textdegree for 10 min. DNA and RNA extractions from 50–100 mg of mouse livers and brains were done using TRIzol Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol.

Expression analysis

Expression of constructs was assessed by staining of lacZ with X-gal as previously described (Portales-Casamar et al. 2010). lacZ staining was performed on a limited number of ear notch samples (Table S1 and Table S2) and on the brains of female mice carrying the Xist deletion. The images of lacZ staining of the mouse brain sections (Figure 1) were adjusted for brightness and contrast using Photoshop, but all images for the same construct (test and control samples) received the same adjustments. For analysis of transcription, ~2 µg of RNA extracted from tissues was converted to cDNA with standard reverse transcription conditions, using M-MLV (Invitrogen) at 42\textdegree for 2 hr followed by a 5-min incubation at 95\textdegree. Quantitative PCR (qPCR) was used to determine relative transcription levels of PHB, HPRT, and the intergenic region between PHB and HPRT compared to Pgk1 in mice carrying the Ple133 construct (NGFR BAC), using a StepOnePlus Real-Time PCR System (Applied Biosystems, Darmstadt, Germany), using Maxima Hot Start Taq (Fermentas) and EvaGreen dye (Biotium). Conditions for qPCR were as follows: 95\textdegree for 5 min; followed by 40 cycles of 95\textdegree for 15 sec, 60\textdegree for 30 sec, and 72\textdegree for 1 min; and a melt curve stage of 95\textdegree for 15 sec, 60\textdegree for 1 min, and an increase of 0.3\textdegree until 95\textdegree. Serial dilutions of genomic DNA from an NGFR female (without the Xist deletion) were used as the standards to which each sample cDNA was compared, to generate a relative quantity of the PHB, HPRT, Pgk1, and intergenic transcription between PHB and HPRT. Expression levels were normalized to Pgk1 expression level, and quantifications were done in triplicate, with any outlier excluded from the analysis. Primer sequences and distances from the assay to the transcription start site are found in Table S3.

DNA methylation analysis

Using the EZ DNA Methylation-Gold Kit or the EZ-96 DNA Methylation-Gold Kit (Zymo Research), 500 ng of DNA obtained from the lysed ear notches and a limited number of liver and brain samples were bisulfite converted, following the manufacturer’s instructions. Internal bisulfite conversion controls were included in the pyrosequencing assays to monitor complete conversion of DNA. Each 25-µl pyrosequencing PCR was performed with 1× PCR buffer (QIAGEN, Valencia, CA), 0.2 mM dNTPs, 0.625 unit Hot Start Taq DNA polymerase (QIAGEN), 0.25 µM forward primer, 0.25 µM reverse primer, and 12–35 ng bisulfite-converted DNA. Assays for CCKBR, ICMT, NOV, and NR2E1 were performed with 0.5 µM forward and reverse primers. Conditions for PCR were 95\textdegree for 15 min, 50 cycles of 94\textdegree for 30 sec, annealing temperature for 30 sec (see Table S3), 72\textdegree for 1 min, and finally 72\textdegree for 10 min. One forward or reverse primer was biotinylated, depending on which strand contained the target region to be sequenced, to subsequently isolate the strand of interest for pyrosequencing. Template preparation for pyrosequencing was done according to the manufacturer’s protocol, using 10–15 µl of PCR products. CDT tips were used to dispense the nucleotides for pyrosequencing, using the PyroMark MD machine (QIAGEN).

Variability in pyrosequencing results within a sample was observed for some DNA, which we attributed to degradation of ear notch DNA that was stored at 4\textdegree for up to 3 years. All promoter assays were replicated at least twice and the average is presented. If the standard deviation of a sample for a particular assay was large enough to be considered an outlier using the modified Z-score method (see below), the data point was not included in the analyses. HPRT, Phf6, and lacZ assays were replicated on sufficient samples that we were confident of their reliability (average standard deviations of 5\%, 3\%, and 5\%, respectively), and therefore for these three assays not all samples were replicated. Each human promoter assay was tested in at least one mouse sample without the target transgene to ensure the specificity of the human primers.

The University of California, Santa Cruz (UCSC) definition and designation of a CpG island (GC content of at least 50\%, length >200 bp, observed\textsubscript{CpG}/expected\textsubscript{CpG} ratio >0.6) were used (Gardiner-Garden and Frommer 1987). The promoter of PHB was classified as a CpG island with intermediate CpG density, which was defined as having a GC content >50\%, an observed\textsubscript{CpG}/expected\textsubscript{CpG} >0.48, length at least 200 bp, and no overlap with the UCSC CpG islands (Weber et al. 2007); the PHB promoter CpG island was located using the CpGIE
Promoter identification of PITX2 (Ple158) was based on the ENCODE chromatin states track downloaded from the UCSC genome browser (Ernst et al. 2011). Repeat masker (http://www.repeatmasker.org, Institute for Systems Biology) and Galaxy (Giardine et al. 2005; Blankenberg et al. 2010; Goecks et al. 2010) were used to determine the base pair composition of LINEs and Alu in the MaxiP constructs.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 5.02. An α-value of 0.05 was used for testing significance. A Mann–Whitney t-test was used to test for significant differences in DNA methylation levels between male and female mice. Mouse strains with modified Z-scores >3.5 in absolute value were considered outliers (Iglewicz and Hoaglin 1993). Spearman’s correlation was performed to examine the relationship between DNA methylation levels of neighboring genes.

**Results**

**DNA methylation reflects XCI of Pleiades constructs**

The Pleiades Promoter Project generated reporter constructs with human promoters and targeted them to the Hprt locus on the mouse X chromosome by homologous recombination (Yang et al. 2009; Portales-Casamar et al. 2010; J.-F. Schmou and E. M. Simpson, unpublished data). Integration of the Pleiades promoter constructs created a chimeric Hprt/Hprt locus that consisted of the human Hprt promoter and exon 1 and mouse Hprt exons 2–9 (Figure 1A). While MinIP constructs contained a human promoter of ≤4 kb in size that drives a reporter (lacZ, EGFP, or EGFP/cre), MaxiP constructs were derived from human BACs of up to 195 kb with the reporter (lacZ or EGFP) inserted at the translation start codon of the gene of interest on the human BAC. To predict whether the constructs were subject to the cis-regulation of XCI now that they were X linked, we generated female mice carrying a deletion at the Xist gene [Xistlox (Csankovszki et al. 1999)] on the X chromosome without the knock-in, thereby causing the Pleiades knock-in to always be on the Xi. The MaxiP constructs AMOTL1 (Ple5), MAOA (Ple127), NR2E1 (Ple142), and NR2F2 (Ple143) were not expressed in the brains of females heterozygous for the Xist deletion, but were expressed in various parts of the brain in females without the Xist deletion (Figure 1B), indicating that these MaxiP constructs are expressed only when present on the Xi and are thus subject to XCI. DNA methylation analysis on DNA from ear notch samples of hemizygous male and heterozygous female mice transgenic for AMOTL1 and NR2E1 showed that CpG island promoters on the BACs were significantly DNA hypermethylated in females compared to males of the same strain (P < 0.05; Figure 1C), in agreement with the XCI statuses assessed by lacZ expression in the females with nonrandom XCI. DNA methylation was also examined in brain and/or liver samples for a few constructs, including the NR2E1 BAC, and similar levels of DNA methylation to those in ear notches were observed (Table S2). We conclude that DNA methylation at CpG island promoters is a reliable predictor of XCI status for transgenes at Hprt. DNA methylation was therefore used to determine the XCI status of the remaining MaxiP constructs (NOV, NGFR, PITX2, and LCT). PITX2 (CpG island 46 designation on UCSC) showed less female DNA methylation than the other MaxiP constructs, which could reflect either the presence or the absence of cis-acting regulators of XCI or a tendency to be preferentially located on the Xi.

To examine the latter possibility we looked at DNA methylation of the flanking Phf6 and Hprt promoters.

**DNA methylation of flanking genes reflects both skewing of XCI and differential capacity for DNA methylation on the Xi**

The Pleiades construct and the human Hprt promoter are located on the same chromosome; therefore, if substantial skewing of XCI were present, their DNA methylation levels would be correlated, reflecting the proportion of cells in which they are both on the Xi. In contrast, Phf6 DNA methylation should not be affected by skewing since it is present on both X chromosomes. The Hprt promoter CpG island was truncated in the chimeric gene, but the chimeric gene complemented the Hprt deletion and provided resistance to HAT selection. Both the Hprt and the Phf6 promoters demonstrated significant DNA hypermethylation in females compared to males (Hprt, female average 38%, male average 5%, P < 0.0001; Phf6, female average 34%, male average 5%, P < 0.0001), suggesting that both neighboring genes were generally subject to XCI. Compared to Phf6 DNA methylation, Hprt showed higher variability in promoter DNA methylation levels between female mice (standard deviations: 10% for Hprt, 4% for Phf6), consistent with variability in levels of skewing of XCI in the samples analyzed. A correlation between the DNA methylation levels at the human promoter and at Hprt, but not with Phf6 was observed (Figure 2), supporting the presence of skewing of XCI in the analyzed ear notch samples. Intriguingly, different MaxiP constructs showed different slopes in the correlation of their DNA methylation level with Hprt (Figure 2B), suggesting that Pleiades promoters have different capacities for DNA methylation when located at the same site on the Xi. To confirm that different constructs had different levels of DNA methylation on the Xi, we analyzed the promoter and Hprt DNA methylation levels in females homozygous for the knock-in and in females heterozygous for the Xist deletion who carry the knock-in solely on the Xi. The AMOTL1, NOV, and NR2E1 MaxiP constructs showed similar levels of Hprt DNA methylation on the Xi (~70%) but slightly different levels of promoter DNA methylation on the Xi (Figure 1D).
DNA methylation levels at PITX2 and NGFR were strikingly different from those at the other MaxiP constructs. PITX2 (CpG island 46) showed a much lower range of DNA methylation when compared to DNA methylation of AMOTL1, NOV, and NR2E1, and DNA hypermethylation of HPRT indicates that the low level of female DNA methylation at PITX2 is not attributable to skewing of XCI but to its intrinsic resistance to accumulate DNA methylation (Figure 2B). In contrast, the NGFR MaxiP construct showed a lower HPRT DNA methylation range (13–33%) compared to the other MaxiP constructs, suggesting that the capacity of HPRT to accumulate DNA methylation is altered in this construct. We also designed a DNA methylation assay ~720 bp downstream of the start codon of the lacZ reporter, which showed similar DNA methylation levels on the Xi for all constructs except the NGFR BAC (Figure S1). The NGFR BAC showed lower levels of HPRT and lacZ DNA methylation on the Xi than expected (HPRT average 41%, outlier; lacZ average 56%), suggesting the region is subject to substantial influence from the genomic context. Therefore, PITX2 showed the largest decrease in capacity to accumulate promoter DNA methylation and the NGFR BAC showed an impact on HPRT DNA methylation. To understand the cis-modulatory effects of the integrated DNA, we explored the PITX2 and NGFR BACs in more detail.

PITX2 is DNA hypermethylated at transcription start sites as well as intragenic and intergenic CpG islands in females

CpG island 46 on the PITX2 BAC is not annotated as the start of the PITX2 transcript, so we analyzed the DNA methylation levels at eight additional locations on the BAC including
exon 1 and intron 2 (non-CpG island), three internal CpG islands, the promoter CpG island of the annotated alternative isoform, and two intergenic CpG islands (Figure 3). Although the first exon does not contain a CpG island, it still showed significantly higher DNA methylation in females than in males ($P = 0.0084$; Figure 3). In fact, all the locations tested in PITX2 generally showed DNA hypermethylation in females compared to males, including the CpG island at the alternative promoter and the intergenic CpG islands. Although our DNA methylation assays are located in the gene body of PITX2, chromatin modifications associated with promoters were found to overlap the assays in intron 2 and CpG islands 46 and 196 (Figure 3) (Ernst et al. 2011), suggesting PITX2 has additional internal promoters. Intergenic CpG islands 59 and 29 show no or very weak chromatin modifications associated with promoters or enhancers, yet both CpG islands showed female-specific DNA hypermethylation (Figure 3). Similarly, analysis of an intergenic and an intragenic CpG island on the NR2E1 BAC demonstrated female-specific DNA hypermethylation (data not shown). Interestingly, lacZ showed a clear difference in DNA methylation levels between males and females (Figure 3), in agreement with the DNA methylation status of multiple sites in PITX2. Thus, while CpG islands 18 and 46 showed lower female DNA methylation (average 14%), because other locations in the gene consistently showed DNA hypermethylation in females at levels consistent with XCI, we conclude that PITX2 is likely subject to XCI based on DNA methylation. Consistent with published data (Straussman et al. 2009), our assessment of male and female blood and lymphoblast lines suggests that in humans the promoter CpG island 196 is always unmethylated while other sites show variable DNA methylation that is not sex specific (data not shown), with the exception of CpG island 29, which appears to be DNA methylated in all tissues except sperm.

![A truncated gene on the NGFR BAC construct partially escapes from XCI](image)

A distinguishing characteristic of the NGFR construct from the other MaxiP constructs was the presence of a truncated gene at the end of the BAC that is adjacent to the HPRT/Hprt locus (Figure 4A). The PHB gene is truncated within the 3′-UTR ~200 bp from the end of the gene, and we hypothesized that PHB escaped from XCI and that the run-on transcription from PHB through the HPRT/Hprt locus positioned ~2.5 kb downstream could be the cause of the reduced HPRT DNA methylation on the Xi. We therefore examined the transcription levels of PHB and the intergenic region between PHB and HPRT/Hprt in males and in females with and without the Xist deletion (Figure 4B). By qPCR, we showed that PHB was not a highly expressed gene relative to Pgk1, but was expressed from the Xi in females heterozygous for the Xist deletion at levels up to 30% of the level of expression observed in males (Figure 4B), while females with random XCI showed a level of PHB expression close to 60% of that in males. Variability was observed in PHB expression levels from the Xi between females, perhaps reflecting the variable escape from XCI previously described for X-linked genes (Carrel and Willard 2005). However, while the expression level at ~1.4 kb downstream of the truncated PHB gene in the intergenic region was essentially the same as at the 3′-UTR of PHB (Figure 4B), this transcription had ceased by ~250 bp upstream of HPRT/Hprt, indicating that there is no substantial run-on transcription through the HPRT/Hprt locus. In addition, analysis of HPRT expression showed that HPRT/Hprt remained inactive on the Xi despite its lower level of DNA methylation and proximity to a gene escaping from XCI. In agreement with the PHB expression analysis, the promoter of PHB has an island of intermediate CpG density (GC% = 52.9, observed/expected CpG = 0.57, length = 1823 bp) that showed relatively low DNA methylation in females with the Xist deletion, but the PHB DNA methylation level on the Xi was still distinct from the level of DNA methylation on the Xa in males (Figure 4C). Overall, it appears that PHB partially escapes from XCI; however, run-on transcription through HPRT/Hprt is not the cause of altered HPRT DNA methylation capacity on the Xi.

**MiniP constructs are generally subject to XCI**

Since our MaxiP results agreed with previous reports that DNA methylation is an accurate marker for XCI status (Goldman et al. 2005), we show that the MiniP data are consistent with XCI on the Xi. The decreased DNA methylation of the BACs (Figure 2A) is likely caused by an increase in DNA methylation in females with the Xist deletion (Figure 2B). Thus, both MaxiP and MiniP constructs show variable DNA methylation that is not sex specific (data not shown), with the exception of CpG island 29, which appears to be DNA methylated in all tissues except sperm.
et al. 1998; Weber et al. 2007), we analyzed promoter DNA methylation of the MiniP constructs to predict their XCI statuses. Heterozygous females overall showed significantly higher DNA methylation levels at promoter CpG islands compared to males (female average, 45%; male average, 12%; \( P < 0.0001 \)). To determine whether there were MiniP promoters that might escape XCI, we analyzed the DNA methylation levels of the constructs separately. DNA methylation levels were analyzed at 46 island-containing MiniP constructs, which originated from 23 human genes. For MiniP constructs that were generated from the same gene and thus shared the same core promoter sequence, the same CpGs were examined for DNA methylation levels. Almost all MiniP constructs showed promoter DNA hypermethylation in females compared to males, with female and male averages of 44% and 4%, respectively, with the outliers removed in the analysis (Figure 5A). Of the Pleiades constructs that were also examined for expression in the ear notch samples, excluding the outliers, all showed female-specific DNA hypermethylation independent of whether the transgenes displayed expression (Figure S2). For three constructs we observed DNA methylation levels <2 standard deviations below the average in a single female, although still >2 standard deviations above the average in males. These females thus might represent a gene with variable inactivation between mice; however, identification of variable escapees is confounded by skewing of XCI that could result in high standard deviations in DNA methylation levels among females of the same strain. The low DNA methylation level in single females for the three constructs was likely attributable to skewing of XCI, since HPRT DNA methylation levels were also lower (8–27%) in these females. Therefore, for a transgene to be qualified as a potential escapee, we required consistent low promoter DNA methylation in multiple heterozygous female mice. Since our MiniP constructs generally showed elevated average DNA methylation in females compared to males, we concluded that none of the MiniP constructs appeared to escape XCI. Promoter DNA hypermethylation was observed in males for the MiniP constructs derived from genes CARTPT, GPX3, ICMT, OXT, and POGZ, but did not appear to correlate with transgene silencing (Table S1 and Figure S2). It is unknown what DNA sequences in these elements generate exceptions to the DNA methylation patterns observed for the majority of the MiniPs, but interestingly for one of these
(CARTPT) the endogenous island shows DNA hypermethylation at the endogenous promoter. In general, we analyzed fewer mice per construct for the MiniPs, but overall MiniP constructs showed higher levels of DNA methylation compared to the MaxiP constructs (average 45% and 33%, respectively, \( P < 0.0001 \)), perhaps reflecting a closer association of the MiniPs with X-linked cis-acting elements or a protective influence of sequences within the large BAC constructs. DNA methylation levels at HPRT and Phf6 were not significantly different between MiniPs and MaxiPs (Figure 5, B and C).

**lacZ reporter consistently reflects DNA methylation pattern of CpG island promoters**

lacZ DNA methylation resembled the DNA methylation pattern of the promoter region in PITX2, leading us to test the utility of lacZ DNA methylation to predict XCI status. Similar to CpG island promoters, female mice showed significantly higher lacZ DNA methylation than males (\( P < 0.0001 \)), even though males did have substantial DNA methylation (male and female average DNA methylation levels of 26% and 49%, respectively). Mice with an autosomal lacZ showed no significant difference in DNA methylation levels between males and females (Figure S3), indicating that the difference in the DNA methylation levels of the X-linked lacZ between the sexes is likely a consequence of the epigenetic regulation of XCI. The lower level of male (Xa) DNA methylation for X-linked lacZ may reflect the previously reported permissive nature of the Hprt integration sites (Bronson et al. 1996; Cvetkovic et al. 2000). Although lacZ showed overall higher DNA methylation than the CpG island promoters (male \( P < 0.0001 \); female \( P = 0.0029 \)), lacZ DNA methylation showed a significant correlation with DNA methylation of the promoter island in females (Figure 6A). Since constructs with and without CpG islands in the promoter both showed a significant difference between female and male lacZ DNA methylation levels (\( P < 0.0001 \) and \( P = 0.0008 \), respectively), we used lacZ DNA methylation as a surrogate for promoter DNA methylation and screened additional Pleiades constructs for which there was no assay for promoter DNA methylation (Figure 6B). Consistent with the lack of lacZ expression in Xist\(^{lox}^{X} /^{X} \text{MAOA}^{X} \) and Xist\(^{lox}^{X} /^{X} \text{NR2F2}^{X} \) mice (Figure 1B), MAOA and NR2F2 showed femalespecific lacZ DNA hypermethylation (Figure 6B), further supporting the usage of this locus as a surrogate to determine XCI status. However, compared to promoter DNA methylation (Figures 1C and 5A), males more often showed DNA hypermethylation of the lacZ reporter (Figure 6B and

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**Figure 4** PHB on the NGFR BAC escapes from XCI. (A) Structure of the NGFR MaxiP construct. PHB is truncated in the 3′-UTR on the BAC, located ~2.5 kb from the HPRT/Hprt locus. Gray and green bars below the MaxiP indicate the locations of the expression and DNA methylation assays, respectively. Internal exons are not shown to scale. (B) Expression of PHB, the intergenic region between PHB and HPRT/Hprt, and HPRT/Hprt (exon 1), normalized to Pgk1. DNA from a mouse targeted at Hprt with BACs for MKI67 (Ple131) and NR2E1 (Ple142) served as negative controls (−) since they lack the PHB gene. The x-axis indicates whether PHB was present only on the Xi or on the Xa in a given mouse, or on either X chromosome (Xa Xi), as in the case for females with random XCI. Error bars indicate ±1 standard deviation between two qPCR runs. (C) DNA methylation of PHB promoter in ear notches of mice carrying the NGFR MaxiP on the Xi, either the Xa or the Xi, or the Xa. Error bars indicate ±1 standard deviation between mice for the strain.
Figure S4). Using the criteria of nonoverlapping standard deviations of DNA methylation between the sexes and a male average DNA methylation level <2 standard deviations of the female average of all strains, we excluded seven strains, including two (DCX Ple53 and VIP Ple250) for which the single male analyzed showed higher DNA methylation than the female average. Thus, we predict an additional 11 constructs subject to XCI based on lacZ DNA methylation.

**Discussion**

Arguably the most dramatic example of cis-regulation in the mammalian genome is silencing of one X chromosome in females. However, the cis-acting elements involved in spreading heterochromatin along the ~155-Mb chromosome from the initiating elements in the X inactivation center remain unknown. Having 74 different human transgenes integrated into the mouse X chromosome presented us with an opportunity to assess cis-regulation of ~1.5 Mb of DNA at the identical genomic location. Analysis of female mice heterozygous for an Xist deletion causing nonrandom inactivation of the knock-in-bearing X chromosome provided clear evidence for XCI of four of the knock-ins (Figure 1). As a more rapid approach to assess the XCI status of multiple transgenes, we used DNA methylation as a surrogate measure of inactivation, since promoter DNA hypermethylation in females relative to males can be attributed to DNA methylation of the Xi and thus reflects inactivation of the gene (Yasukochi et al. 2010; Cotton et al. 2011). We demonstrated that in addition to such DNA hypermethylation for the genes subject to XCI, the escaping PHB gene in our system exhibited low promoter DNA methylation in both sexes, validating the usage of DNA methylation as a indicator of XCI.

**Figure 5** Promoter DNA methylation of the MiniP constructs is reflective of XCI. (A) DNA methylation levels of individual MiniP constructs. Constructs with lacZ, EGFP, and EGFP/cre as the reporter are colored in blue, green, and black, respectively. Circles, DNA methylation of the individual sample; bar in the x-axis of the error bars, average DNA methylation for the strain; error bars, ±1 standard deviation between mice for the strain; shaded regions, 2 standard deviations from the average DNA methylation level with outlier strains removed. Outliers are marked with asterisks (*). Female outlier: POGZ (Ple167). Male outliers: CARTPT (Ple20, Ple21), GPX3 (Ple97), ICMT (Ple123), OXT (Ple152, Ple153), and POGZ (Ple167, Ple170). Modified Z-scores >3.5 in absolute values were marked as outliers (Iglewicz and Hoaglin 1993). (B and C) Phf6 promoter (B) and HPRT promoter (C) both showed a significant difference in DNA methylation levels between males and females. A Mann-Whitney t-test was used to test for significance. Boxplot whiskers are 5th–95th percentiles. Circles, the average DNA methylation levels of each strain.
methylation to detect genes subject to, and escaping from, XCI. As the determination of whether a gene is subject to XCI could be confounded by skewing of XCI and there can be variability between females for whether a gene escapes XCI (Carrel and Willard 2005), we included an assessment of HPRT DNA methylation to detect samples with skewed XCI and we required consistently high promoter DNA methylation in multiple females to call a gene subject to XCI. Overall, 92% of the constructs analyzed showed DNA hypermethylation of the human promoters in female mice compared to males (Figures 1C and 5A), indicative of XCI of the knock-in gene.

Our goal in determining the XCI status of the 74 knock-in constructs was to characterize cis-acting elements involved in the spread of epigenetic silencing. In 1983 Gartler and Riggs proposed waystations as elements enriched upon the X chromosome that aid in spreading the silencing signal along the chromosome, based on the limited spread of XCI into autosomes that is seen for X;autosome translocations (Gartler and Riggs 1983). Because MiniP constructs are small transgenes, it is not surprising that all were shown to be subject to XCI, since they are in close proximity to X-linked DNA and putative waystations (Figures 1C and 5A), indicative of XCI of the knock-in gene.

Our goal in determining the XCI status of the 74 knock-in constructs was to characterize cis-acting elements involved in the spread of epigenetic silencing. In 1983 Gartler and Riggs proposed waystations as elements enriched upon the X chromosome that aid in spreading the silencing signal along the chromosome, based on the limited spread of XCI into autosomes that is seen for X;autosome translocations (Gartler and Riggs 1983). Because MiniP constructs are small transgenes, it is not surprising that all were shown to be subject to XCI, since they are in close proximity to X-linked DNA and putative waystations. Indeed, the majority of previous studies on X-linked transgenes have reported silencing of the examined transgenes on the Xi. However, the ~187-kb chicken transferrin transgene is one of the few exceptions that consistently escaped from XCI (Goldman et al. 1987, 1998). As the MaxiP constructs were of a similar size (120–195 kb), we anticipated that the MaxiPs originating from autosomes would have a high probability to lack waystations and escape from XCI. However, our results demonstrated that mouse XCI is consistently capable of inactivating foreign transgenes up to 195 kb. Thus, escape from XCI of the chicken transgene may reflect integration into a waystation-poor region relative to HPRT. In addition, however, there is now evidence for a different type of cis-regulatory element. Recently four different integrations of a Kdm5c BAC recapitulated both escape from XCI for Kdm5c and silencing for the flanking Tspyl2 and Iqsec2 genes, strongly supporting the existence of a cis-acting element on the BAC that controlled escape from XCI (Li and Carrel 2008). Therefore, we hypothesize that there are both waystations and escape elements regulating the spread of XCI.

Escape elements are presumably outside the promoter as none of the 46 MiniPs examined in this study nor the majority of previously examined transgenes escape from XCI. We determined that the PHB gene escapes from XCI, and as waystations are reduced in abundance on autosomes and NGFR is subject to XCI while being farther from the mouse X-linked DNA than PHB (see Table S2), we conclude that the PHB region likely carries an escape element to escape from XCI. The observed reduction of HPRT DNA methylation to 41% on the Xi when adjacent to PHB, from an average of 70% DNA methylation on the Xi for other MaxiP constructs, suggests that the dominant escape element also influenced the HPRT locus. The PHB gene is truncated in the 3′-UTR of the gene; however, we demonstrated that transcription ceased before the HPRT promoter, and since the promoter, splice junctions, and coding sequences are intact, we do not believe that this truncation per se affects either the propensity for PHB to escape from XCI or the loss of

Figure 6  lacZ DNA methylation can be used as a surrogate for promoter DNA methylation. (A) Spearman’s correlation between lacZ and promoter DNA methylation levels in females. Circles, DNA methylation levels from an individual mouse. (B) lacZ DNA methylation levels of the Pleiades constructs that do not have a DNA methylation assay in the promoter region due to difficulty in assay design, assay failure, or absence of a CpG island. All constructs shown here have lacZ as the reporter on the X chromosome. Circles, DNA methylation levels from an individual mouse; bar in the center of the error bars, average DNA methylation for the strain; error bars, ±1 standard deviation between mice for the strain; shaded regions are the 2 standard deviations from the female average DNA methylation level with the outlier strains removed. Outliers are marked with asterisks (*). Female outlier: VIP (Ple250).
DNA methylation at HPRT. Interestingly, the reduced DNA methylation at HPRT was still sufficient to maintain XCI, while the 15% DNA methylation of PHB was insufficient for silencing, although there was not full expression from the Xi relative to the Xa. Given that we observed only one such escape element in 47 genes and 1.5 Mb of DNA, our data support the existence of relatively rare dominant escape elements. As escape from XCI is frequent in X;autosome translocations (~30% (reviewed in Yang et al. 2011)), it is likely that, as previously proposed, such escape more generally reflects a lack of waystations rather than the presence of escape elements. We therefore decided to examine the large MaxiP constructs to determine whether there was evidence for particular elements that might be functioning as waystations.

Waystations have been proposed to be repetitive elements, and we calculated the base coverage in the MaxiP for several repeat elements previously positively or negatively correlated with genes escaping XCI: LINE-1, LINE-2, and Alu (Bailey et al. 2000; Carrel et al. 2006; Wang et al. 2006). Only Ple142 (NR2E1) and Ple126 (LCT) appeared to possess an environment that resembles the genomic context of escape elements based on LINE-1, LINE-2, and Alu base coverage on the BAC (Figure S5). However, promoter DNA hypermethylation of NR2E1 and LCT in females suggests that the genes were subject to XCI (Figure 1C). Although only eight BACs were examined, our analysis of LINES and Alu content suggests that these three repetitive elements are insufficient to determine whether a gene is subject to XCI and that additional repeats or a combination of other factors may be required to provide a better prediction of the XCI status.

In addition to our search for cis-acting regulatory elements, our analysis of the Pleiades human knock-in constructs into the X-linked Hprt docking site revealed several other insights into the relationship of DNA methylation with XCI. First, we demonstrated that constructs have an intrinsic differential capacity for DNA methylation. Through analyzing MaxiP DNA methylation of female mice with complete nonrandom XCI due to an Xist deletion, we showed that different MaxiP constructs could accumulate DNA methylation at the promoters to different extents on the Xi (Figure 2B). However, the HPRT promoter and the lacZ reporter, which are shared among the MaxiP constructs, generally exhibited similar levels of DNA methylation (Figure S1), suggesting that the capacity to accumulate DNA methylation is a characteristic of the DNA sequence. Intriguingly, there may be differences between hemizygous and heterozygous or between homozygous and heterozygous states, as the observed promoter DNA methylation levels in females with the Xist deletion tend to be lower than the expected level of DNA methylation on the Xi based on the assumption that the DNA methylation on the Xa is equivalent between males and females.

Second, the differential female:male DNA methylation observed on the X is found beyond CpG-island promoters. Xa-specific DNA methylation has been reported in gene bodies (Hellman and Chess 2007). Our results demonstrated that the influence of XCI on DNA methylation of transgenes applies not only to promoters, but also to gene body and intergenic CpG islands, since all analyzed locations on the PITX2 BAC showed female-specific DNA hypermethylation that was not observed on the endogenous human chromosome (Figure 3), including regions such as CpG island 29 for which there is no evidence for promoter activity (Ernst et al. 2011) or overlap with conserved transcription factor binding sites (TRANSFAC Biobase, http://www.gene-regulation.com/pub/databases.html). Therefore, it is possible that the default state of CpG islands on the X chromosome is to acquire DNA methylation on the Xi and this is independent of whether the transgenes were expressed (Figure S2), consistent with the majority of the CpG islands being DNA hypermethylated on the Xi (Cotton et al. 2011; Sharp et al. 2011), and tissue-specific genes such as the human X-linked androgen receptor showing female-specific DNA hypermethylation independent of expression (Allen et al. 1992).

The recognition of CpG islands for DNA methylation on the Xi could explain the DNA hypermethylation in females compared to males on the X chromosome for the lacZ reporter (Figure 6), which is essentially an ~3000-bp CpG island. Promoter-less artificial CpG islands inserted into the 3′UTR of an autosomal and an X-linked gene have been shown to recruit the unmethylated CpG-binding protein Cfp1 and the promoter histone mark H3K4me3 even in the absence of RNA polymerase II binding (Thomson et al. 2010), although the X-linked locus has some DNA methylation presumably due to XCI. The ability of CpG-rich sequences to acquire characteristics of promoters further supports using lacZ DNA methylation as a surrogate for promoter DNA methylation to predict whether transgenes are subject to XCI. However, lacZ DNA methylation is not as robust a predictor of XCI status as the promoter DNA methylation since a higher frequency of males with DNA hypermethylation was observed.

A third insight was the unexpected observation that the differential female:male DNA methylation may reflect not only gain of DNA methylation on the Xi, but also protection from DNA methylation on the Xa. In general, promoter CpG islands are unmethylated on the autosomes; however, ~4% are reported to show DNA methylation, often with variability between tissues (Shen et al. 2007). Four of the 35 autosomal CpG islands analyzed (CARTPT, OXT, THY1, and PITX2: CpG island 29) showed an average of >20% DNA methylation in male and female cell lines and/or blood samples (data not shown; Straussman et al. 2009). However, when THY1 and the PITX2 BAC were present on the X chromosome, they became unmethylated on the Xa; this loss of DNA methylation on the Xa compared to the autosomal locus was also observed for a non-CpG island site (exon 1 of PITX2) and the lacZ reporter. In the knock-in mice, CARTPT continued to show DNA methylation; however, OXT dropped from 60% DNA methylation on the autosome to only 20% DNA methylation in males, again showing decreased DNA methylation in males. In general we observed a dominant regulation of XCI on promoter DNA methylation,
with female-specific gain of DNA hypermethylation and in several cases a male-specific loss of DNA methylation.

Overall, our analysis of the Pleiades human promoter constructs integrated into the mouse Hprt locus identified 1 of 47 genes in >1.5 Mb of human DNA that escaped inactivation. We propose that there is a dominant cis-acting escape element near the PHB gene that allows it to escape from XCI in an otherwise inactivated region on the X chromosome. This element exerts an influence on the DNA methylation of the flanking HPRT locus, but does not lower DNA methylation to a level that allows expression from the Xi. That eight autosomal BACs ranging in size from 120 to 195 kb contained a gene subject to XCI when integrated at the Hprt site suggests that waystations are likely able to act over a distance of at least 100 kb, in the absence of dominantly acting escape elements. Further analyses of BAC integrations at the Hprt locus will be useful to identify the nature of these escape elements as well as the boundaries that prevent their influence on adjacent genes.

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Targeting of >1.5 Mb of Human DNA into the Mouse X Chromosome Reveals Presence of cis-Acting Regulators of Epigenetic Silencing

Christine Yang, Andrea J. McLeod, Allison M. Cotton, Charles N. de Leeuw, Stéphanie Laprise, Kathleen G. Banks, Elizabeth M. Simpson, and Carolyn J. Brown
Figure S1  DNA methylation of HPRT and lacZ was high on the Xi except for NGFR (Ple133). Spearman correlation between HPRT and lacZ DNA methylation levels was significant for NR2F2 (Ple143: r=0.7545; p=0.0368), but not for MAOA (Ple127). DNA methylation levels of NGFR in Xist<sup>lox</sup> females (encircled on the graph) were substantially lower than AMOTL1, NOV, and NR2E1 in Xist<sup>lox</sup> females. Filled circles, DNA methylation in individual female mice heterozygous for the knock-in and carrying wild-type Xist on both X chromosomes. Triangle, DNA methylation of the knock-in on the inactive X chromosome in females heterozygous for an Xist deletion (Xist<sup>del</sup>). DNA methylation levels in four mice homozygous for the MAOA MaxiP were also examined (open circles).
Figure S2  Promoter DNA methylation of the Pleiades constructs is reflective of XCI and independent of expression. Females generally showed DNA hypermethylation compared to males regardless of expression status of the transgene. Expression status (expressed and non-expressed) was based on lacZ staining in the ear notches of mice. Circles, DNA methylation of the individual sample; bar in the center of the error bars, average DNA methylation for the strain; error bars, ± one standard deviation between mice for the strain. Outliers in DNA methylation are marked with asterisks (*). Female outlier: POGZ (Ple167). Male outliers: ICMT (Ple123), OXT (Ple152, Ple153), POGZ (Ple167, Ple170). Modified Z-score greater than 3.5 in absolute values were marked as outliers (Iglewicz and Hoaglin 1993).
Figure S3  DNA methylation of autosomal lacZ reporter was not significantly different between the sexes. Three categories of mice with an autosomal lacZ reporter at the ROSA26 locus (Gt(ROSA)26Sor<sub>tm1Sor</sub>/J; Soriano 1999) were assessed. Category 1: lacZ is activated by EGFP/cre driven by different X-linked MiniPs. Category 2: lacZ had been previously activated by cre. Category 3: lacZ expression did not required activation by cre (Friedrich and Soriano 1991). Each circle represents the level of DNA methylation in an individual mouse. In all categories the autosomal lacZ was driven by the same promoter. Bar, average; error bars, ± one standard deviation between mice for the strain. Significance was tested using Mann-Whitney t-test; n.t., not tested.
Figure S4  *lacZ* reporter in constructs that were analysed for promoter DNA methylation generally showed DNA hypermethylation in females compared to males. Circles, DNA methylation of the individual sample; bar in the center of the error bars, average DNA methylation for the strain; error bars, ± one standard deviation between mice for the strain.
Figure S5  MaxiPs, while all subject to XCI, showed no correlation between the XCI status and repeat contents. Repeat content for LINE-1 (A), Alu (B), and LINE-2 (C) did not correlate strongly with the XCI statuses of the MaxiPs. Genes that are subject to XCI are predicted to be enriched in LINE-1 and LINE-2 and depleted in Alu, while genes that escape from XCI are predicted to be depleted in LINE-1 and LINE-2 and enriched in Alu. The median levels of repeat content in 100-kb windows surrounding genes subject to XCI (red line) and genes escaping from XCI (green line) were estimated from Wang et al. (2006).
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Table S1  Summary of MiniP constructs

| Gene name | Ple#  | Reporter   | Promoter size (kb) | Average female promoter methylation (%) | Average male promoter methylation (%) | lacZ expression | Promoter CpG island |
|-----------|-------|------------|--------------------|----------------------------------------|--------------------------------------|-----------------|---------------------|
| ATP6V1C2  | Ple7  | EGFP       | 2.4                | 32                                     | 6                                    | N/A             | 119                 |
| CARTPT    | Ple20 | EGFP       | 3.6                | 44                                     | 23                                   | N/A             | 63                  |
| CCKBR     | Ple21 | lacZ       | 2.4                | 47                                     | 62                                   | NT              | 26                  |
|           | Ple24 | lacZ       | 3.7                | 64                                     | 4                                    | +               |
|           | Ple25 | lacZ       | 3.6                | 65                                     | 5                                    | NT              |
| CLDNS     | Ple34 | lacZ       | 3.5                | 43                                     | 2                                    | +               | 147                 |
| DRD1      | Ple61 | EGFP       | 3.7                | 37                                     | 2                                    | N/A             | 145                 |
|           | Ple62 | EGFP       | 3.4                | 41                                     | N/A                                  | N/A             |
| FEV       | Ple66 | EGFP       | 1.5                | 37                                     | N/A                                  | N/A             | 126                 |
|           | Ple67 | lacZ       | 2.2                | 43                                     | 5                                    | N/A             |
| GPX3      | Ple97 | EGFP       | 3.4                | 59                                     | 19                                   | N/A             | 43                  |
|           | Ple98 | EGFP       | 2.9                | 47                                     | 3                                    | N/A             |
| GRP       | Ple99 | EGFP/cre   | 1.4                | 75                                     | 7                                    | NT              | 57                  |
|           | Ple100| EGFP       | 2.4                | 52                                     | 5                                    | N/A             |
| HAP1      | Ple103| EGFP/cre   | 0.7                | 26                                     | 2                                    | NT              | 130                 |
|           | Ple104| EGFP       | 2.1                | 50                                     | 1                                    | N/A             |
|           | Ple106| EGFP       | 1.1                | 36                                     | 1                                    | N/A             |
| HBEGF     | Ple107| EGFP       | 3.4                | 47                                     | 3                                    | N/A             | 64                  |
|           | Ple108| EGFP       | 3.6                | 61                                     | 2                                    | N/A             |
|           | Ple109| EGFP       | 3.5                | 54                                     | 4                                    | N/A             |
| HTR1A     | Ple119| lacZ       | 3.6                | 47                                     | 6                                    | -               | 100                 |
| ICMT      | Ple123| lacZ       | 2.2                | 66                                     | 53                                   | +               | 74                  |
| NR2E1     | Ple140| lacZ       | 3.9                | 46 (ear) 45 (brain) 32 (liver)         | 12 (ear) 10 (brain) 9 (liver)         | +               | 318                 |
| NTSR1     | Ple144| EGFP       | 3.0                | 36                                     | 2                                    | N/A             | 158                 |
|           | Ple146| EGFP       | 3.8                | 46                                     | 3                                    | N/A             |
|           | Ple147| EGFP       | 2.1                | 57                                     | 5                                    | N/A             |
| OLIG1     | Ple148| EGFP       | 0.9                | 37                                     | 3                                    | N/A             | 202                 |
|           | Ple150| EGFP       | 2.9                | 38                                     | 2                                    | N/A             |
|           | Ple151| EGFP       | 2.6                | 30                                     | 1                                    | N/A             |
| OXT       | Ple152| EGFP       | 1.3                | 73                                     | 24                                   | N/A             | 105                 |
|           | Ple153| lacZ       | 2.7                | 68                                     | 25                                   | +               |
| PITX3     | Ple160| EGFP/cre   | 2.5                | 42                                     | N/A                                  | NT              | 104                 |
|           | Ple161| EGFP/cre   | 3.9                | 28                                     | 4                                    | NT              |
|           | Ple162| EGFP/cre   | 3.6                | 47                                     | 6                                    | NT              |
| POGZ      | Ple167| EGFP/cre   | 1.2                | 97                                    | 71                                   | +               | 48                  |
|           | Ple168| EGFP       | 4.0                | 54                                     | 3                                    | N/A             |
|           | Ple170| EgFP       | 3.2                | 17                                     | 22                                   | N/A             |
|           |       | lacZ       |                    | 44                                     | 30                                   | +               |
| RLPB1L2   | Ple179| lacZ       | 3.5                | 42                                     | 6                                    | NT              | 59                  |
|           | Ple180| lacZ       | 3.8                | 36                                     | 5                                    | -               |
|           | Ple181| lacZ       | 3.7                | 41                                     | 6                                    | NT              |
All average DNA methylation levels refer to DNA methylation examined in the ear notch samples only, except for NR2E1 where DNA methylation was also examined in brain and liver. The numbers indicated for the promoter CpG island refer to the UCSC CpG island designations. *full-length CpG island; †detected as outliers in levels of DNA methylation in males and/or females; NT, not tested; N/A, not applicable.

| SLC6A4 | Ple198 | EGFP | 2.6 | 37 | 6 | N/A | 81 |
|--------|--------|------|-----|----|---|-----|----|
| TAC1   | Ple214 | EGFP | 2.7 | N/A| 7 | N/A | 137|
|        |        | lacZ | 31  | N/A|   | NT  |    |
|        | Ple217 | EGFP | 0.9 | 25 | 7 | N/A |    |
| THY1   | Ple229 | EGFP | 1.2 | 52 | N/A| N/A | 48 |
|        |        | lacZ | 37  | 5  |   | NT  |    |
| UGT8   | Ple241 | EGFP | 3.1 | 30 | 2 | N/A | 121|
|        | Ple242 | EGFP | 2.5 | 32 | 3 | N/A |    |
Table S2  Summary of MaxiP constructs

| Gene name | Ple# | BAC size (kb) | Minimal distance from TSS to X boundary* (kb) | **Average X**<sub>ist</sub><sup>lox</sup> female promoter methylation (%) | **Average female promoter methylation (%)** | **Average male promoter methylation (%)** | **lacZ expression in ear** | Promoter CpG island<sup>b</sup> | Other CpG islands<sup>c</sup> | Other transcripts on BAC<sup>d</sup> |
|-----------|------|---------------|-----------------------------------------------|---------------------------------|---------------------------------|---------------------------------|-----------------------------|------------------|------------------|------------------|
| AMOT1     | Ple5 | 193.7         | 80.5                                          | 50                              | 29                              | 3                              | +                          | 114              | +                | -                |
| MCM6      | Ple126 | 138.6        | 24.2                                          | ND                              | 49                              | 4                              | N/A                        | 91               | -                | +                |
| NGFR      | Ple133 | 157.9        | 78.1                                          | 69 #                            | 43                              | 4                              | -                          | 255              | +                | +                |
| NOV       | Ple134 | 184.3        | 59.8                                          | 66                              | 38                              | 3                              | -                          | 57               | -                | -                |
| NR2E1     | Ple142 | 120.2        | 55.2                                          | 58                              | 39 #                            | 9 #                            | -                          | 318              | +                | +                |
| PHB       | Ple142 | 157.9        | 20.3                                          | 14 #                            | 9                               | 3 N/A                          | ^                          | +                | +                |
| PITX2-exon1 | Ple158 | 182.2        | 82.1                                          | NT                              |                                  | 40                             | 7                          |                  |                  |                  |
|          |       |               |                                               |                                 |                                  | 14                             | 4                          |                  |                  |                  |
|          |       |               |                                               |                                 |                                  | 26                             | 6                          |                  |                  |                  |
|          |       |               |                                               |                                 |                                  | 14                             | 4                          |                  |                  |                  |
|          |       |               |                                               |                                 |                                  | 44                             | 4                          |                  |                  |                  |
|          |       |               |                                               |                                 |                                  | 40                             | 6                          |                  |                  |                  |
|          |       |               |                                               |                                 |                                  | 46                             | 3                          |                  |                  |                  |
|          |       |               |                                               |                                 |                                  | 42                             | 5                          |                  |                  |                  |
|          |       |               |                                               |                                 |                                  | 35                             | 8                          |                  |                  |                  |

*MCM6 is a gene on the LCT BAC and the results of MCM6 are shown in the table. The table shows DNA methylation levels in ear notch samples only. The constructs indicated with # showed similar DNA methylation levels in brain and/or liver to ear notch samples. TSS, transcription start site; * shortest distance from TSS to mouse X chromosomal DNA, which could be from the TSS to mouse Hprt locus or from the TSS to the intergenic region between Hprt and Phf6; <sup>b</sup> presence of CpG island overlapping TSS; <sup>c</sup> presence of non-promoter associated CpG island(s) on the BAC, as defined by UCSC; <sup>d</sup> presence of other TSS that is not associated with the gene of interest on the BAC, as defined by UCSC; ND, not done; NT, not tested; N/A, not applicable. The numbers indicated for the promoter CpG island refer to the UCSC designation for CpG islands. ^, no CpG island found at PHB promoter according to UCSC definition of islands, but there is an island of intermediate CpG density.
| Assay         | Construct       | Distance of assay from TSS (bp) | Sequence (5'–3') | Ta (°C) | Position of CpGs analysed |
|--------------|----------------|---------------------------------|------------------|---------|---------------------------|
| AMOTL1_F1    | Ple 5          | 72 (5')                         | GGGATAAAGGAGGAGGTGGT | 55      | 8-13                      |
| AMOTL1_R1    | (RP11-936P10)  |                                 | *TCACCTAAAAACCCTACTCCACC |         |                           |
| AMOTL1_S2    |                |                                 | GGAGGGTTTTGTTAGA   |         |                           |
| ATP6V1C2_F1  | Ple 7          | 544 (5')                        | AGGTGGGAGGTGTTAGGTAAT | 53.9    | 1-5                       |
| ATP6V1C2_R1  |                |                                 | *CAAAAAAACCTACTCCCTAAAAATCT |         |                           |
| ATP6V1C2_S1  |                |                                 | GGAGGGTTTTGTTAGA   |         |                           |
| CARTPT_F1    | Ple 20-21      | 296 (5')                        | GTAAATGTGGTGGTTAGGTAAT | 55      | 1-3                       |
| CARTPT_R1    |                |                                 | *TCCCAACCTCTAAATATAAAACT |         |                           |
| CARTPT_S1    |                |                                 | TGGGTGGTGGTTTAGA    |         |                           |
| CCKBR_F1     | Ple 24-25      | 424 (5')                        | GAGGAGTTGAGGGAATTAA | 55      | 1-5                       |
| CCKBR_R1     |                |                                 | *AATACTTTAATCTAACTAAAACC |         |                           |
| CCKBR_S1     |                |                                 | GAGGAGTTGAGGGAATTAA |         |                           |
| CLDN5_F1     | Ple 34         | 820 (3')                        | AGTTGGTAGGTTTTTGATTGTG | 53.3    | 1-5                       |
| CLDN5_R1     |                |                                 | *AAAAATACCTCTTAAATTTTC |         |                           |
| CLDN5_S1     |                |                                 | GGGGTAGGGTTTTTGTA    |         |                           |
| DRD1_F1      | Ple 61-62      | 38 (3')                         | TATTTGTATAGGTTTTTGAGGTTAAT | 53.3    | 1-5                       |
| DRD1_R1      |                |                                 | *CCTTCAACCTCAACAAACAA |         |                           |
| DRD1_S1      |                |                                 | ATTGTTATAGGTTTTTGAGA |         |                           |
| FEV_F1       | Ple 66-67      | 1 (3')                          | *GGAGGGGAGGAGAGTTGA | 53.9    | 1-4                       |
| FEV_R1       |                |                                 | CCCTCCCTAAAAACCTCTTCTCTC |         |                           |
| FEV_S1       |                |                                 | AAAACCTTCTTCAA      |         |                           |
| GPX3_F1      | Ple 97-98      | 39 (3')                         | TGGGGAGAAGGGAATTAGT | 55      | 1-5                       |
| GPX3_R1      |                |                                 | * CCAAACCACTCTTCAACCA |         |                           |
| GPX3_S2      |                |                                 | GGGAGGAGGGAATTAGT   |         |                           |
| GRP_F1       | Ple 99-100     | 216 (5')                        | AGAGGGAGGAGTTTATTAATATTGTGTTT | 55      | 1-5                       |
| GRP_R1       |                |                                 | *CATTCCCCCTTTTTTCTTT |         |                           |
| GRP_S1       |                |                                 | AAATTTGTTGGAGTAGGA  |         |                           |
| HAP1_F1      | Ple 103-104,   | 262 (5')                        | GGAGGGTTTGTGGGTTAGG | 53.9    | 1-4                       |
|             | 106            |                                 | *ATTTTTTCTACCTCTTCTCCCTCTCC |         |                           |
| HAP1_S1      |                |                                 | GTTGTGTTTAGGGAATTTTTAAGTGAAT | 55      | 1-5                       |
| HBEFGc_F1    | Ple 107-109    | 209 (5')                        | GTTTGGGGAAGGTTAGGAAT | 55      | 1-5                       |
| HBEFGc_R1    |                |                                 | *TCACAATTTTTAAAAACCAAAC |         |                           |
| HBEFGc_S1    |                |                                 | GTTTGGGGAAGGGTTA    |         |                           |
| HPRTb_F1     | All constructs | 94 (5')                         | GGAATAGGGAGTTTTTTGAAATAGG | 55      | 1-3                       |
| HPRTb_R1     |                |                                 | *CCTACCAATTTTTAAAAACTCAAATAAATA |         |                           |
| HPRTb_S1     |                |                                 | GGGAGGGAGGAGGTTA    |         |                           |
| HTR1A_F1     | Ple 119        | 266 (5')                        | *TTTGGGATGGAGGATTTTGT | 55      | 5-8                       |
| Gene/Rec | Position/Range | Functional Annotation | β-Actin Sites | 58.3% | 1-4
|----------|----------------|-----------------------|--------------|-------|---
| HTR1A_R1 | Ple 123-124    | 206 (5')              | ACTCCAACTAAAAAAAAACTAAATTAACCT | CTAAAAAECTAAAATTAACC | 58.3 | 1-4
| HTR1A_S1 |               |                       | GGAATTTTTGGAGTTTGGGATTA | *CATCCAACCTCTAAAAAACACTAAAA | 55   | 1-4
| ICMT_F1  | Ple 126        | 190 (5')              | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 3-7
| ICMT_R1  | (RP11-406M16)  |                       | CCTCTAAAAAAAAACCTACTACCTTT | TGGTAAAGTGAAGTTGGAGTTATTG | 55   | 1-3
| ICMT_S1  |               |                       | GTAAGGGTAAAGTTTGTATTTTGT | *TTTCACCCCTACATAAAAAACCCTTATTTG | 55   | 1-4
| LacZ-meth_F1 | Reporter | ~724 (3' of lacZ seq) | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 1-4
| LacZ-meth_R1 |          |                       | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 1-4
| LacZ-meth_S1 |            |                       | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 1-4
| MCM6_F1  | Ple 134       | 26 (5')               | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 1-4
| MCM6_R1  | (RP11-158L10) |                       | *CCCAAAAAACCTAAAAACCAACCT | GTGAAGGTTAGTTGGATA | 48   | 1-6
| MCM6_S1  |               |                       | *CCCAAAAAACCTAAAAACCAACCT | GTGAAGGTTAGTTGGATA | 48   | 1-6
| mPhf6_F1 | Endogenous    | 305 (5')              | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 1-4
| mPhf6_R1 |               |                       | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 1-4
| mPhf6_S1 |               |                       | GGAATTTTTGGAGTTTGGGATTA | *CATCCAACCTCTAAAAAACACTAAAA | 55   | 1-4
| NGFR_F1  | Ple 133       | 279 (5')              | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 1-4
| NGFR_R1  | (RP11-158L10) |                       | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 1-4
| NGFR_S1  |               |                       | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 1-4
| NOV_F33  | Ple 134       | 26 (5')               | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 1-4
| NOV_R33  | (RP11-840I14) |                       | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 1-4
| NOV_S1   |               |                       | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 1-4
| NR2E1_F1 | Ple 140, 142  | 22 (3')               | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| NR2E1_R1 | (RP11-144P8)  |                       | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| NR2E1_S1 |               |                       | AATCTTAAAAAAACCTACTACCTTT | TGGGAATTAGTTGGATA | 55   | 2-4
| NR2E1-CpG40_F1 | Ple 140, 142 | Intragenic | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| NR2E1-CpG40_R1 | (RP11-144P8) | | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| NR2E1-CpG40_S1 | | | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| NR2E1-CpG75_F1 | Ple 140, 142 | Intergenic | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| NR2E1-CpG75_R30 | (RP11-144P8) | | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| NR2E1-CpG75_S1 | | | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| NTSR1_F1 | Ple 144,       | 368 (5')              | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| NTSR1_R1 | 146-147        |                       | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| OLI1_F1  | Ple 148,       | 36 (5')               | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| OLI1_R1  | 150-151        |                       | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| OLI1_S1  |               |                       | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| OXT_F1   | Ple 152-153    | 107 (5')              | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| OXT_R1   |               |                       | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| OXT_S1   |               |                       | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| PHB-IC_F1 | Ple 133       | 1 (5')                | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| PHB-IC_R1 | (RP11-158L10) |                       | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4
| PHB-IC_S1 |               |                       | *GTGGAATGTATTTAAGATATTTGAAAA | TGGGAATTAGTTGGATA | 55   | 2-4

12 SI C. Yang et al.
PITX2-CpG18_F1  Ple 158  Gene body  GGGATTGGGGTTAATTAGTTTTTGG  58.3  1-4
PITX2-CpG18_R1  (RP11-26811)  1385 (5') of Alt-TSS  *AACCTCCCTCCCCCTCAAAATTCT
PITX2-CpG18_S1  Ple 158  Gene body  *AAATTTGATGTTTTTTGAAGGGTTTT  58.3  1-3
PITX2-CpG22_R1  (RP11-26811)  18 (3') of Alt-TSS  ACAAATATAACATTTCCCTAAAATA
PITX2-CpG22_S1  Ple 158  Gene body  AACTAATAAATTTCCCTACCTCC
PITX2-CpG29_F1  Ple 158  Intergenic  *GTTTTGATTTGGAGGAGTTAGGT  58.3  1-5
PITX2-CpG29_R1  (RP11-26811)  18 (3') of Alt-TSS  AACCTAACCACCAATCTCC
PITX2-CpG29_S1  Ple 158  Gene body  AACCTAACCACCAACCA
PITX2-CpG46_F92  Ple 158  Gene body  GTATTTTTTTAGTTTTGTTTGAGTAGAG  48  1-5
PITX2-CpG46_R92  (RP11-26811)  18 (3') of Alt-TSS  *CCCCAACCACAAATCTTTTT
PITX2-CpG46_S1  Ple 158  Gene body  TGGTGGAGAGGGGAG
PITX2-CpG59_F1  Ple 158  Intergenic  TGATTTAGGTTTTTTTTGTATGAATT  55  1-7
PITX2-CpG59_R1  (RP11-26811)  18 (3') of Alt-TSS  *CCATACATTAAACAAAACACTAATT
PITX2-CpG59_S1  Ple 158  Gene body  GGATTTTTTGGATTTTATGA
PITX2-CpG100b_F1  Ple 158  Gene body  TGGAGTGGAAAAAGTGTGTTATA  56.3  1-6
PITX2-CpG100b_R1  (RP11-26811)  18 (3') of Alt-TSS  *AACCTAAATAAATACAAATAAACCTAAT
PITX2-CpG100b_S1  Ple 158  Gene body  GTGGAAAAGTGTGTTATA
PITX2-CpG196b_F1  Ple 158  Gene body  TGGTTTTAAAGATGTAGTTAGTTATA  55  1-6
PITX2-CpG196b_R1  (RP11-26811)  18 (3') of Alt-TSS  *ACTCAACTCCAAACACCCAAA
PITX2-CpG196b_S1  Ple 158  Gene body  GATTTTAGTTGTTAGGGGAG
PITX2-exon1_F1  Ple 158  Gene body  *AAAGTTAGGAGGATTAATATATAGGT  58.3  1-3
PITX2-exon1_R1  (RP11-26811)  18 (3') of Alt-TSS  ACTTCCCTCTCTAAACAAATTTTCT
PITX2-exon1_S1  Ple 158  Gene body  ACTTCCCTCTCTAAACAAAT
PITX2-intron2_F1  Ple 158  Gene body  AGATATTAATAATTTTATAGGGTGTTAA  53.3  1-3
PITX2-intron2_R1  (RP11-26811)  18 (3') of Alt-TSS  *AACCTTTTATACCAACACCTTTT
PITX2-intron2_S1  Ple 158  Gene body  TAATTTATAGGGTGTTGAGG
PITX3_F1  Ple 160-162  Gene body  GAGTTTTAGTTAGGTTAGGTAAG  55  1-9
PITX3_R20  Ple 160-162  Gene body  *CCATCCACTTTAACAACCAA
PITX3_S1  Ple 160-162  Gene body  GTAGGGTTTGGTAGTTATGTA
POGZ_F1  Ple 167-168, 84 (5')  Gene body  GTAGGGTTTGGTGAGGTTAT  55  1-4
POGZ_R1  Ple 167-168, 84 (5')  Gene body  *CCTTTTACACCTCCCCAATTA
POGZ_S1  Ple 167-168, 84 (5')  Gene body  GGGTTTGGAGGGTTTGA
RLBP1L2b_F1  Ple 179-181  Gene body  TGGGGAGGTGGGAAATAGT  58.3  1-5
RLBP1L2b_R1  (RP11-26811)  18 (3') of Alt-TSS  *CCCCACTCCCTACAAACTACT
RLBP1L2b_S1  Ple 198  Gene body  GGGGAGGTGGGAAAG
SLC6A4_F1  Ple 198  Gene body  *TGTAGGTTTTAGGAAAGAAAGAGAGA  58.3  6-10
SLC6A4_R68  Ple 198  Gene body  CATCCTACCTTTACTCTTTAATAACTCAAAAAACAAAT
SLC6A4_S1  Ple 198  Gene body  GAAATTTAATGTTGTTATGATAGG
TAC1_F1  Ple 214, 217  Gene body  GAATTTAATTGTTTATGATAGG
TAC1_R1  Ple 214, 217  Gene body  *TTTAATATACCCCCCTTCTCTTTCTTTT

C. Yang et al. 13 SI
| Gene      | Primer Location | Primer Sequences | Ta, Ta1 | Alt-TSS | SNP |
|-----------|-----------------|------------------|--------|---------|-----|
| TAC1_S1   | Ple 229         | GGGTTTAGATGGTTATGGGTA | 58.3   | 1-3     |     |
| THY1_F1   |                  | GGAGGTGGGTTTTAGTTGAAA |        |         |     |
| THY1_R1   |                  | *AAAAACATTATATATCCCTCTCCCTAAA |        |         |     |
| THY1_S1   | Ple 241-242     | GTGGGTGGTGGTAGAAG | 58.3   | 1-4     |     |
| UGT8_F1   |                  | *CCCACCTTCTCCCTTTTA |        |         |     |
| UGT8_R1   |                  | TGGGTGGGTGATAGA |        |         |     |
| UGT8_S1   |                  |                    |        |         |     |
| qhHPRT_F2 | All constructs  | CCTGCTTCTCCTCACGTTCAG | 60     | Expression |     |
| qhHPRT_R2 |                  | CGGGAAAGCCGAGAGTT |        |         |     |
| qHPRT-5'F1| All constructs  | CAAATCTCCTGGCATCACATACC | 60     | Expression |     |
| qHPRT-5'R1|                  | AGTGCCCACGACATAGTGGT |        |         |     |
| qHPRT-5'B1| All constructs  | GCCACAGGTAGTGCAAGGTCTT | 60     | Expression |     |
| qHPRT-5'B_R1|                | CCAGTCATCGGTAATCTT |        |         |     |
| qPgk1-e1_F1| Endogenous     | CGTCTGCCGCGGCTGGT | 60     | Expression |     |
| qPgk1-e1_R1|                | AACACCGTGGCGCGGAG |        |         |     |
| qPHB-3'F1  | Ple 133         | CTGTCATGATGGGAAGGTTCG | 60     | Expression |     |
| qPHB-3'F1  | (RP11-158L10)   | AGGCCTGCGCCTCGATCCA |        |         |     |
| mFln_F6   | Endogenous      | *CCAGCTTCCCTAGTCCAAATGC | 58.3   | SNP     |     |
| mFln_R3   |                  | TGCATACAGTACGTCTCAGTACAA |        |         |     |
| mFln_S1   |                  | CCTAGAGAGGGCTGAA |        |         |     |

Primers biotinylated at the 5' end are indicated with an asterisk (*). Position of analysed CpGs is relative to the sequencing primer. Distance of pyrosequencing assays from transcription start site (TSS) is the distance of the closest CpG to the TSS. Ta, annealing temperature for PCR; Alt-TSS, alternative transcription start site; SNP, single-nucleotide polymorphism.