Supplemental Text

Supplemental Text S1. Opossum Fetal Development.

Opossum fetuses are born on embryonic day 14. It is sometimes stated that this corresponds to a mouse 11-12 day fetus, but this comparison is primarily a convenience. In fact, opossum ontogeney does not precisely parallel that of rodents (Smith 2001). More precisely, the newborn opossum has anatomical features that are more advanced, and others that lag behind those of the 11-12 day mouse. For example the jaws and front limbs are strongly developed, which enables the newborn to crawl unaided from the birth canal to the region of the teats where it attaches to a nipple; the female gonads are largely undifferentiated and meiosis has not begun; the nervous system is rudimentary and still capable of remarkable regeneration; and hind limbs are small and paddle-like (See Samollow 2008 for further details). For this reason it is more accurate to view the day-14 (newborn) opossum roughly equivalent to an 11-12 day mouse fetus in overall development, but distinct from it in specific ways. Developmental progression of the opossum, from conception through parturition, has been described in great detail by Mate et al. (1994). More general considerations of marsupial development have been reviewed by Selwood et al. (1997), Behringer et al. (2006), and Selwood and Johnston (2006).

Supplemental Text S2. Effective X-linked Hemizygosity in Female Marsupials: Implications and Solutions.

In this study, we found that opossum *Rsx* is a paternally expressed (maternally imprinted) gene not only in EEM but also in fetal brain. For all other the annotated opossum X-linked genes examined, all non-escaper genes are 100% imprinted with monoallelic, maternal expression, while escaper genes exhibit biallelic, but unequal expression of both alleles, with all but two showing preferential expression from the maternal allele. The opossum X thus acts as a chromosome-wide paternal imprinting cluster with a small minority of genes escaping imprinting and one maternally imprinted gene (*Rsx*) in both EEM and somatic tissues. As a result, the female is effectively hemizygous for the majority of X-linked genes, as is the male. This condition will manifest the presence of deleterious recessive mutations on the Xm in all female cells, greatly reducing fitness in females, just as in males. By contrast, rXCI in the somatic tissues of eutherian mammals confers some of the advantages of heterozygosity because females express both parental alleles at X-linked loci, albeit individually in different cells. Through rXCI, dosage compensation between the two sexes is achieved without sacrificing many of the advantages of diploidy. The theoretical population genetics of this problem has been investigated, and conditions for invasion of random XCI into a population with imprinted XCI is clearly favored under conditions that depend on dominance and the degree of sex-specific selection (Connallon and Clark 2013).

However, not all opossum X-linked genes are subject to pXCI. It could be argued that the escaping status of some genes is the derived state, because ~85% of X-linked

genes have 100% monoallelic maternal expression and most escaper genes show preferential maternal expression. In view of the fact that most of these genes also show greater total expression in females than in males, such leaky expression might have been established by selection to maintain some elevated level of gene activity at these particular loci in females. By expressing both alleles, the hemizygosity problem is solved; and, by selectively escaping X-inactivation, escaper genes could be upregulated in total expression levels in females. Nevertheless, analysis of synonymous and non-synonymous substitution rates in primate X-linked genes led Park et al. (2010) to the conclude that human escaper genes have been under strong purifying selection relative to non-escaper genes and that selection was driven largely by escapers with Y-linked homologues, possibly due to novel selective constraints arising from functional divergence of the X and Y homologues at these loci. In this regard it is noteworthy that there is almost no overlap between the opossum pXCI escapers and human/mouse rXCI escapers (Supplemental Table 8) (Carrel and Willard 2005; Yang et al. 2010), which could be due to pXCI escapers of marsupials facing different selection pressures from those that impinge upon rXCI escapers of eutherian mammals.

Supplemental Text S3. Predicting XCI status using escaper/non-escaper histone state characteristics.

In this study, ~56% (176 of 312) genes of the expressed X-linked loci examined had informative SNPs in the reciprocal crosses we employed, and among these we discovered 24 pXCI escaper genes in E13 fetal brain and EEM. These escaper genes share four

major characteristics: 1. By definition, they display biallelic expression in allele-specific RNA-seq and pyrosequencing results; 2. The H3K27me3 marks are depleted across the entire gene body, consistent with biallelic expression; 3. Enrichment of H3K4me3 peaks is significantly higher for escaper genes than for non-escapers; and 4. Almost all escaper genes show significantly higher expression in females relative to males. Applying the second, third, and fourth characteristics (H3K27me3 peak coverage < 5%, H3K4me3 fold-enrichment > 8, and F/ME ratio > 1.05), we searched among 136 expressed X-linked genes without informative SNPs, and identified 11 candidate escaper genes based on these criteria (Supplemental Table S7 and Figures S32, S36, S42-S45). Three of these candidate escapers were confirmed as genuine escapers in fibroblast cell lines that were heterozygous for informative SNPs (data not shown). Appropriate SNPs have not been identified for the remaining eight candidate escaper genes. Assuming these 11 genes to be representative, the success of this preliminary exploration implies that epigenetic characteristics can be powerful predictors of the expression states of marsupial X-linked genes.

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