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Molecular control of the oocyte to embryo transition

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The elucidation of the molecular control of the initiation of mammalian embryogenesis is possible now that the transcriptomes of the full-grown oocyte and two-cell stage embryo have been prepared and analysed. Functional annotation of the transcriptomes using gene ontology vocabularies, allows comparison of the oocyte and two-cell stage embryo between themselves, and with all known mouse genes in the Mouse Genome Database. Using this methodology one can outline the general distinguishing features of the oocyte and the two-cell stage embryo. This, when combined with oocyte-specific targeted deletion of genes, allows us to dissect the molecular networks at play as the differentiated oocyte and sperm transit into blastomeres with unlimited developmental potential.

Keywords: oocyte; embryo; transcriptome; reprogramming; retrotransposons

1. INTRODUCTION: INITIATION OF MAMMALIAN DEVELOPMENT

Because of the scarcity of mammalian preimplantation embryos and their difficulty of access, molecular studies of early mammalian development have been limited to querying whether transcripts or protein products of specific genes are present in oocytes and preimplantation embryos (Suzumori *et al.* 2003; Wu *et al.* 2003). To solve this problem, several groups have attempted to capture the total molecular information within these stages by making cDNA libraries. Sequences of several cDNA libraries of ovulated oocytes and preimplantation embryos have been made available to the scientific community so that the presence of specific genes of interest can be identified (Rothstein *et al.* 1992; Sasaki *et al.* 1998; Ko *et al.* 2000). We have taken a systems biology approach, attempting the following: (i) to identify the transcriptomes, or at least a significant statistical sampling of gene transcripts, of these early stages; (ii) to outline the molecular processes controlling progression through the oocyte to embryo transition; (iii) to determine when the embryonic genome is activated and what the first gene products are; and (iv) to understand the function of single genes and/or networks of genes as they become activated.

2. EPIGENESIS VERSUS PREFORMATION

Oocyte maturation, and transition of the completely differentiated gametes to a totipotent embryonic cell in mice, as in other metazoans, occur in the absence of new transcription (Davidson 1986). Consequently, completion of the meiotic cell cycles, the first mitotic cell cycles, reprogramming of the genomes contributed by the egg and the sperm, and activation of the embryonic genome itself, relies on transcripts and proteins made during oocyte growth and on the waves of signal transduction associated with oocyte maturation, ovulation and fertilization. Successful development rarely occurs when nuclei from adult or later embryonic cells are transferred into an ovulated oocyte, a fault attributed to aberrant reprogramming of these differentiated nuclei in the oocyte cytoplasm (Rideout *et al.* 2001). However, the mechanism by which the haploid genomes of the differentiated sperm and egg are reprogrammed during normal embryogenesis is also unclear. The maternally derived histones and their chaperones, which mediate the transfer of core histones to DNA, influence nucleosome assembly (Kikyo & Wolffe 2000), while acetylation of the core octamer histones also has a major influence on activity/silencing of transcription (Thompson *et al.* 1998). In special cases, methylation is also important in setting bounds on gene activity (Walsh & Bestor 1999).

Because there is no transcription, the molecular changes necessary to accomplish this change in structure and state depend on: (i) the stability and differential translation of maternal mRNAs stored in the oocyte cytoplasm; (ii) the stability of the proteins made in the oocyte; (iii) their post-translational processing; and, perhaps, (iv) generation of the first embryonic transcripts. It is here that the discussion between epigenesis and preformation was first begun (Pinto-Correia 1997) and, until the relevant mechanisms are unfolded, it is here that this debate will continue. The materials necessary to initiate development are present in the egg and may be set in motion without fertilization *but* the interaction with the sperm and its genetic contribution is required for successful reprogramming and development. We suggest that genetic reprogramming is a stage-specific process, an intricate part of the genomic network at the outset of development.

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One contribution of 14 to a Discussion Meeting Issue 'Epigenesis versus preformation during mammalian development'.

3. A SYSTEMS APPROACH TO THE OOCYTE TO EMBRYO TRANSITION

Some time ago, our laboratory prepared large and representative cDNA libraries from mouse early embryos (Rothstein *et al.* 1992) and, with our materials and advice, John Eppig of The Jackson Laboratory prepared a similar library from full-grown oocytes. Now that the complete mouse genome is sequenced and assembled, and most of the genes have been named or noted, we have annotated and analysed sufficient sequences from these libraries to identify genes that are abundant in the full-grown oocyte; just after maternal transcription ceases, and those still present just before whole-scale activation of the newly formed embryonic genome, in the two-cell stage (A. V. Evsikov, unpublished data; Evsikov *et al.* 2003). Fifteen thousand to twenty thousand cDNAs were sequenced, the ESTs were assembled, and BLASTN searches of GenBank and the public Mouse Genome Assembly have been performed. From this information, we have been able to identify approximately 5500 individual genes expressed in the full-grown oocyte library and 4000 in the two-cell embryo (A. V. Evsikov, unpublished data; Evsikov *et al.* 2003). The function of approximately half of them is completely unknown. Interestingly, *ca.* 10% of the genes in the full-grown oocyte library, but less than 5% of the two-cell stage library, seem to be unique to oocytes and preimplantation stage embryos, suggesting that the oocyte to embryo transition is accomplished by interactions of a few stage-specific gene products with many proteins active in other cell types.

To determine whether the cDNA libraries were an adequate representation of the transcripts in oocytes and embryos, we examined the presence of a large number of these genes in full-grown oocytes, ovulated oocytes and preimplantation stage embryos by Northern blot analysis (Oh *et al.* 2000), RT–PCR analysis (Evsikov *et al.* 2003) and by quantitative dot blot analysis (Wang *et al.* 2001; de Vries *et al.* 2003). Their patterns of expression match their presence in the library. A few transcripts that are not present in the ESTs, but that are known to be expressed at very low levels in oocytes and embryos, have been detected in the original cDNA libraries by PCR.

Library sequence analysis provides a list of the genes expressed in a cell type sampled, and gene ontology vocabularies (Gene Ontology Consortium 2001) enable functional annotation of the transcriptome in question. Such analyses allowed us to group genes of similar function so that the physiology of the two-cell stage could be better understood. Our analysis indicated an elevated expression of genes involved in cell cycle regulation, protein modification and degradation, and translational regulation/RNA metabolism in the two-cell embryo. Furthermore, transcripts of genes encoding receptors and ligands were underrepresented in the two-cell stage embryo (Evsikov *et al.* 2003).

(a) *Cell cycle regulation*

This new information matches the accumulating body of information regarding the cell biology of oocytes and two-cell embryos. The meiotic and first mitotic cell cycles in the oocyte to embryo transition are long and exquisitely regulated. During the transitions from the full-grown oocyte to fertilizable (metaphase II-arrested) oocyte to fertilized zygote, oscillations in MPF activity are a striking example. Cyclin B1, a regulatory subunit of MPF, is translated in a regulated fashion from stored mRNA (Tay *et al.* 2000) to substitute for cyclin B2, which rapidly degrades when MPF activity ceases after meiotic metaphase I (Kobayashi *et al.* 1991). Failure to degrade cyclin B2 leads to oocyte arrest at metaphase I, as shown in experiments where proteasome function is inhibited during oogenesis (Josefsberg *et al.* 2000). CDK1, a catalytic subunit of MPF, is stable in the oocyte (Hampl & Eppig 1995), but its dephosphorylation/phosphorylation correlates with the oscillatory changes in MPF activity. Thus, timely degradation of cyclin B2, translation of cyclin B1 and changes in phosphorylation of CDK1 provide a mechanism for oocyte re-entry into metaphase II without an interphase. The long time-span of the first three cell cycles of the mouse embryo tempts the suggestion that they are controlled by negative regulators. Indeed, in the two-cell transcriptome such genes are among those most abundantly expressed (Evsikov *et al.* 2003).

(b) *Protein degradation*

To foster the change essential for the navigation of the oocyte to embryo transition, both the transcripts and proteins present must change in composition and abundance. In preliminary analysis the profile of abundant transcripts changes during the oocyte to embryo transition so that those encoding oocyte-specific products, such as the zona pellucida specific proteins (ZP2, ZP3), are abundant in the full-grown oocyte but are absent from the two-cell stage embryo (A. V. Evsikov, unpublished data). Not only does transcript abundance change, but the proteins active in the full-grown oocyte, such as tissue-specific plasminogen activator, are no longer active in the two-cell embryo (Salles *et al.* 1992). What are the mechanisms by which protein degradation is accomplished? F-box proteins, which serve as receptors to recruit different substrates such as β-catenin or cyclins to the SCF protein–ubiquitin ligase complexes, as well as members of the ubiquitination–proteasome pathway, are highly abundant in full-grown oocytes and two-cell embryos. Transcripts for components of the proteasome itself are abundant, transcripts and products for components of the 19S and 20S subunits are detected in the oocyte and preimplantation embryo. Thus, both the mechanism for targeting proteins for destruction and the cellular components necessary for rapid protein turnover are present. Although we have not made a full-scale and direct study of the abundance and timing of post-translational processes, some proteins, such as the UBE2I component of the ubiquitination pathway and the 19S proteasome subunits, change from nucleus to cytoplasm during negotiation of the oocyte to embryo transition. These changes in intracellular localization may result from differences in post-translational processing of the individual components and/or their molecular interactions as the oocyte to embryo transition proceeds.

(c) *Translation regulation and RNA metabolism*

Differential mRNA stability is one important means to regulate the availability of transcripts for translation. Some mRNAs transcribed in the growing oocyte are deadenylated (to *ca.* 20 nucleotides) and stored in the ooplasm for later translation. At the time of translation, between one

and several hundred adenine residues are added to the limited 3′poly(A) tail (Bachvarova 1992; Sheets *et al.* 1994; Stebbins-Boaz *et al.* 1996). The two *cis*-elements required for this interaction are the nuclear polyadenylation signal AAUAAA, and the CPE, (A)UUUU(U)UA(A)U (Fox *et al.* 1989; McGrew *et al.* 1989). A disproportionately high number of transcripts in the two-cell library translated during the oocyte to embryo transition contain CPE-specific sequences in the 3′UTR (Oh *et al.* 2000). On oocyte maturation and re-entry into meiosis in *Xenopus*, the poly(A) tails of mRNAs encoding MOS (moloney sarcoma oncogene; proto-oncogene serine/threonine-protein kinase MOS), several cyclins and CDK2 are elongated and these proteins are translated. Deadenylated mRNAs of immature oocytes are bound by protein complexes containing the CPEB, maskin and the 5′ cap-binding factor eIF4E. As a result of the progesterone surge that induces oocyte maturation, CPEB is phosphorylated by the serine/threonine kinase Aurora. CPEB is then free to interact with the cleavage and polyadenylation specificity factor bound to the nuclear polyadenylation signal AAUAAA, recruiting poly(A) polymerase to form the active polyadenylation complex (Mendez *et al.* 2000*a*,*b*). Maskin subsequently dissociates from eIF4E, allowing binding by eIF4E of other 5′ cap elements, assembly of the 48S initiation complex and initiation of translation (Cao & Richter 2002; Groisman *et al.* 2002). Polyadenylation thus signifies that active translation, through interaction of a complex of factors that forms at the 5′ cap structure and near the poly(A) tail, is taking place (Richter & Theurkauf 2001; Darnell 2002; Maniatis & Reed 2002). Programmed translation provides a mechanism for an orderly change of the molecular environment within the cell. CPEB null mutant females lack oocytes, thus demonstrating the essential role of CPEB in oogenesis (Tay & Richter 2001). CPEB and a mouse orthologue of maskin are abundant transcripts in the two-cell cDNA library (Evsikov *et al.* 2003).

AREs are 3′UTRs characterized by repetition of one or more AUUUA motifs within an AU-rich background sequence. AREs control developmental stability and/or degradation of mRNAs after fertilization in *Xenopus* (Chen & Shyu 1995; Voeltz & Steitz 1998) and are known to regulate message deadenylation and degradation for a number of mammalian genes (Chen & Shyu 1995). Many of the mRNAs in the FGO and two-cell cDNA libraries contain putative AREs $(AUUUA)_3$. The libraries also contain transcripts encoding putative homologues of the *Xenopus* embryonic poly(A)-binding protein, which binds AREs and regulates deadenylation in eggs (Voeltz *et al.* 2001). Transcripts of Elavl1, Elavl2 and Elavl3, whose protein products are known to bind mammalian AREs, are also found in both libraries. Future analysis will reveal whether the presence of the ARE motifs is predictive of the changing abundance of cDNAs that we detect during the oocyte to embryo transition.

Other 3′UTR elements, such as the putative embryonic CPEs, polyuridine ($U_{12\text{-}27}$) and polycystine (C_{14}) (Wu *et al.* 1997; Paillard *et al.* 2000), as well as the EDEN motif (Bouvet *et al.* 1994) have been shown to regulate message stability in other species, principally *Xenopus*. In *Xenopus* it has been suggested that polyuridine is bound by ElrA, a member of the ELAV family, to prevent polyadenylation until after fertilization (Wu *et al.* 1997), whereas polycystine is thought to be bound by α-CP2, preventing default deadenylation and enhanced translation after fertilization (Paillard *et al.* 2000). As with the AREs, these 3′UTR motifs have yet to be systematically examined in transcripts available in the oocyte and two-cell embryo, although transcripts of homologues of the putative binding proteins, such as α-CP2, are present in the libraries (A. V. Evsikov, unpublished data; Evsikov *et al.* 2003).

(d) *Receptors and ligands*

Very early embryos are independent of environmental signals and are characterized by autoregulation and self-sufficiency, as demonstrated by their ability to develop normally in a chemically defined medium, so a relative decrease in the abundance of receptor and ligand-encoding transcripts in them versus somatic cell types is appropriate. Combining a conditional oocyte-specific *cre*-recombinase/*loxP* null-mutagenesis strategy (de Vries *et al.* 2000), with transcriptome analysis, we began investigating the roles of cell adhesion and signalling in oogenesis and early embryogenesis. The first gene we focused on was the homophilic cell-adhesion molecule E-cadherin. Subsequently we analysed its intracellular partner β-catenin, a molecule that is also the linchpin in the canonical Wnt signalling pathway.

E-cadherin transcripts are very rare in the two-cell cDNA library; the transcript contains a CPE, and is apparently polyadenylated during the oocyte to embryo transition (Oh *et al.* 2000). The protein is detectable on the surface of the oocyte and preimplantation embryo (Ohsugi *et al.* 1996). E-cadherin null mutant embryos cannot form the proper intercellular contacts necessary to produce a functional blastocyst (Larue *et al.* 1994). Oocyte-specific elimination of E-cadherin transcripts does not affect fecundity or fertility of females mated to wild-type males (de Vries *et al.* 2003). The behaviour of two- to eight-cell embryonic blastomeres is phenotypically deviant, in that they do not adhere to each other. However, transcription from the paternal genome, which is activated at the four-cell stage, gives rise to sufficient E-cadherin to promote compaction about a cell cycle late, in the 16-cell embryo. Delaying compaction by a cell cycle has no apparent effect on subsequent embryogenesis (de Vries *et al.* 2003).

A balance between tethered and free β-catenin is required to maintain cellular homeostasis. The N-terminal third of this multi-functional molecule contains binding sites for both E-cadherin and α-catenin, which effectively bridges E-cadherin interactions at the cell surface with the actin cytoskeleton (Aberle *et al.* 1994). The N-terminal third of the molecule also contains binding sites for molecules involved in the control of the cytoplasmic concentration of β-catenin. Once bound by a multi-protein complex containing APC, axin, GSK3β and CKIα, it is phosphorylated and marked for ubiquitination and degradation (Liu *et al.* 2002). Wnt ligand interactions with the frizzled receptors activate dishevelled receptors to displace axin from this multi-protein complex, releasing free non-phosphorylated β-catenin, which translocates to the nucleus and binds to members of the TCF/LEF family of transcription factors (Kishida *et al.* 1999*a*,*b*; Salic *et al.* 2000). In the nucleus, this complex can activate new gene

transcription from specific gene promoters by binding the transcriptional coactivators p300 and CBP (Hecht *et al.* 2000) and/or by binding BRG1, a component of the SWI/SNF and Rsc chromatin remodelling complexes (Nielsen *et al.* 2002). The p300/CBP and BRG1 binding sites are located in the C-terminal portion of β-catenin.

We eliminated the N-terminal portion of the β-catenin protein ($^{-}$N-β-catenin) from oocytes and early embryos by oocyte-specific (*Zp3-cre*) removal of floxed exons 2–6. This part of β-catenin contains all or a portion of the binding sites for E-cadherin, α-catenin, axin, APC, GSK3β, CKIα and TCF/LEF. Blastomeres derived from these mutant females crossed to wild-type males, like those derived from E-cadherin mutant females, do not adhere to each other at the two- and four-cell stage. Once protein synthesized from the intact β-catenin paternal allele, which is transcribed from the late two-cell stage, is detectable at the four-cell to eight-cell transition, adhesion is restored, allowing compaction at the eight-cell stage. Because $^{-}$N-β-catenin mice are still able to produce the carboxy portion of the molecule representing exons 7–13 and containing the binding sites for BRG1, and CBP/p300, we are re-engineering our construct to determine whether β-catenin plays a part in chromatin remodelling at the activation of the embryonic genome.

$^{-}$N-β-catenin mutant mice have been shown by others to be defective in Wnt signalling (Huelsken *et al.* 2000, 2001; Brault *et al.* 2001). Examination of the FGO and two-cell libraries for expression of frizzled receptors or Wnt ligands revealed that there are no members abundant enough to be seen in the FGO, although frizzled 5 is present at low transcript numbers in the two-cell stage. This result, though preliminary, is in concert with the hypothesis that the oocyte to embryo transition is accomplished without the traditional receptor–ligand interactions that characterize somatic cell interactions with their milieu and with other cells.

In contrast to females lacking E-cadherin, $^{-}$N-β-catenin mutant females produce significantly fewer offspring than control mice (de Vries *et al.* 2003). We are now investigating whether alterations in the Wnt-signalling pathway during oogenesis may explain the lower number of offspring of these mutant females. Expression of members of the Wnt and frizzled gene families have been demonstrated in mammalian ovaries, though no definitive exploration of which cell type expresses these proteins has been undertaken (Ricken *et al.* 2002).

When we combine this functional investigation with the knowledge from the transcriptome of the full-grown oocyte, two-cell embryo and blastocyst, a picture of the molecular networks controlling gene expression at the outset of embryogenesis emerges (figure 1).

4. STAGE-SPECIFIC EXPRESSION OF RETROTRANSPOSONS

Barbara McClintock's view of transposons was that they were controlling elements (Comfort 2001). Active retrotransposons, located in the 3′ or 5′UTR of genes, can affect the transcription of adjacent genes by producing sense or antisense transcripts of those genes. Indeed, a recent study found evidence for both activation and silencing of neighbouring genes following transcriptional activation of the mobile retrotransposon Wis2-1A in wheat. Transcriptional activation of an LTR in the same orientation as a neighbouring gene may activate or maintain its transcription, whereas if the LTR is in the opposite orientation antisense transcripts may contribute to gene silencing (Kashkush *et al.* 2003).

Transcription of repetitive elements is known to change as the methylation state of the elements changes, with their highest expression in mice at the nadir of net genome methylation in the preimplantation embryo (Yoder *et al.* 1997). The methylation status of at least some of these elements may be dependent on the parent of origin, for example, L1 elements are methylated in the male gamete, whereas they are unmethylated in the growing oocyte (Sanford *et al.* 1987). Class III endogenous retroviruses, including foamy virus-like (ERV-L) elements, and the MaLR elements, MT and ORR1, are extremely abundant in the mouse genome and, in contrast to humans, are still active (Smit 1999; Mouse Genome Sequencing Consortium 2002).

Despite a similarity in their heritage and structure, we have found stage-specific transcription of these class III elements. *MT transposon-like elements* are abundantly expressed in the unfertilized egg, and they are also capable of integrating into adjacent genes, perhaps changing the level of the protein product in the oocyte and early embryo (A. E. Peaston, A. V. Evsikov, W. N. de Vries, A. Holbrook and B. B. Knowles, unpublished data; Evsikov *et al.* 2003). By contrast, the *ORR1 transposon-like elements* and the *mouse endogenous retrovirus L* appear to be expressed at the beginning of the two-cell stage and a very high level of reverse transcriptase activity is found in the late two- to eight-cell stage embryo (Evsikov *et al.* 2003). This, together with the finding that genes expressed at the two-cell stage are significantly more likely to have pseudogenes than those whose expression is limited to somatic tissues (Evsikov *et al.* 2003), suggests that these mobile elements are currently changing the mouse genome.

The active *class II retroviruses*, the *IAPs* and the *early-transposons*, form a large proportion of the two-cell and blastocyst transcriptomes, respectively (Evsikov *et al.* 2003). Stage-specific expression of *IAP* transcripts of different subtypes has been observed in growing oocytes, zygotes through to early cleavage stage embryos, and in late cleavage stage embryos through to blastocysts (Piko *et al.* 1984). *IAP* transcripts are relatively scarce in the full-grown oocyte; this has been attributed to *de novo* cytosine methylation of *IAP* DNA initiated in growing oocytes (Walsh *et al.* 1998) and may reflect DNA methylation associated with general transcriptional silence at this time. The majority of *IAP* DNA in blastocysts is methylated (Walsh *et al.* 1998), whereas the methylation status of *IAP* DNA in embryo stages between zygote and blastocyst is unclear. These class II retroviruses account for over 10% of spontaneous mouse mutants (Hamilton & Frankel 2001) and their imprinting and mosaic silencing are known to mediate somatic phenotypic variation epigenetically in mice (Whitelaw & Martin 2001).

The initiation of targeted heterochromatin formation and transcriptional repression at sites of repetitive element transcription in eukaryotic organisms requires the RNAi machinery (Jenuwein 2002; Stevenson & Jarvis 2003).

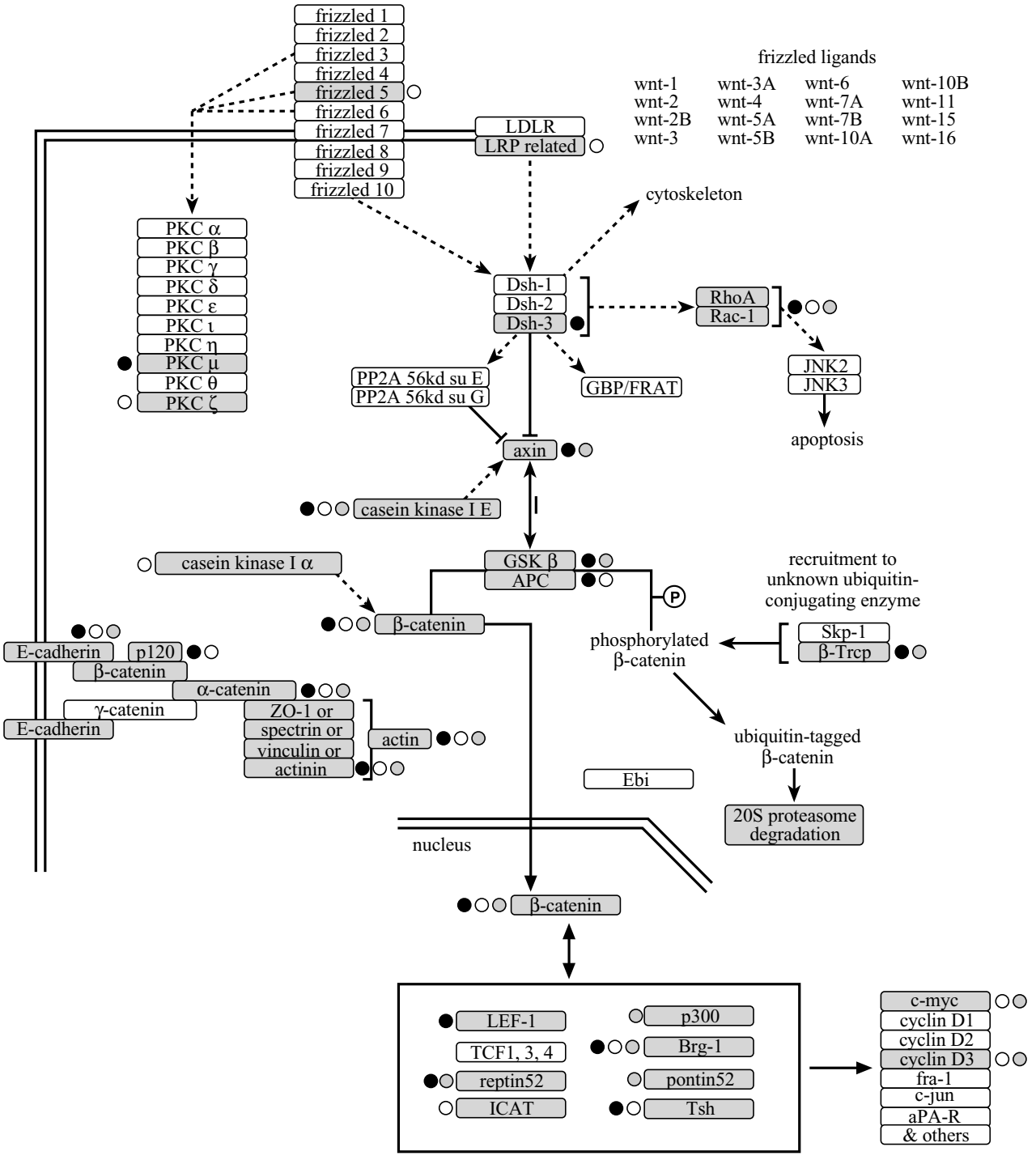

Figure 1. Schematic representation of the canonical Wnt pathway depicting the presence of ESTs in the FGO, two-cell and blastocyst libraries. The molecules that are present in one or more libraries are indicated by shaded boxes. The libraries they are present in are indicated by dots: black circle, FGO; white circle, two-cell; grey circle, blastocyst. Note the absence of Wnt ligands in all three libraries.

Indeed, RNAi may be required for post-translational silencing of repetitive elements in animal cells (Hammond *et al.* 2001). Experimental evidence suggests that RNAi is functional in oocytes (Svoboda *et al.* 2000) and we have found that *eIF2C2* and *Dicer1*, two of the few known mammalian elements of RNAi machinery, are more abundant in the two-cell embryo cDNA library than in any other (Evsikov *et al.* 2003). This raises the possibility that in the early embryos RNAi, perhaps in response to some of these mobile elements, is involved in the epigenetic restructuring of the mammalian embryonic genome.

This leads us to suggest that retroviral elements may play a part in shaping stage-specific gene expression in the gametes and early embryos. A similar suggestion has been proposed, that transposable long interspersed nuclear elements may facilitate X-chromosome inactivation (Lyon 1998; Bailey *et al.* 2000). This is an area of considerable theoretical interest with little experimental underpinning. The stage-specific abundance of transcripts representing a particular type of element may provide an indication for the understanding of various modifications of the genome as it progresses from germ cell to embryo.

5. CONCLUSIONS

Combining the transcriptome with functional assays, and with the knowledge of the pathways in which specific molecules participate, gives access to the molecular networks controlling the activation of the embryonic genome. The mysteries of molecular reprogramming are inextricably bound to understanding the factors influencing histone acetylation and DNA methylation and nucleosome formation. Whether endogenous retro-elements contribute to the overall pattern of gene transcription at the initiation of development awaits the outcome of explicit experimental designs.

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GLOSSARY

APC: adenomatous polyposis coli
ARE: AU-rich elements
CBP: CREB-binding protein
CKIα: casein kinase Iα
CPE: cytoplasmic polyadenylation element
CPEB: cytoplasmic polyadenylation element binding protein
EST: expressed sequence tag

FGO: full-grown oocyte
GSK3β: glycogen synthase kinase 3β
IAP: intracisternal-A particle
LEF: lymphoid enhancer binding factor
LTR: long terminal repeat
MPF: M-phase promoting factor
RNAi: RNA interference
SCF: Skp1-Cullin-F-box
TCF: T-cell factor (transcription factor)
UTR: untranslated region