

# RNA transcript profiling during zygotic gene activation in the preimplantation mouse embryo

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## Abstract

Zygotic gene activation is essential for development beyond the 2-cell stage in the preimplantation mouse embryo. Based on  $\alpha$ -amanitin-sensitive BrUTP incorporation, transcription initiates in the 1-cell embryo and a major reprogramming of gene expression driven by newly expressed genes is prominently observed during the 2-cell stage. Superimposed on genome activation is the development of a transcriptionally repressive state that is mediated at the level of chromatin structure. The identity of the genes that are expressed during the 1- and 2-cell stages, however, is poorly described, as are those genes involved in mediating the transcriptionally repressive state. Using the Affymetrix MOE430 mouse GeneChip set, we characterized the set of  $\alpha$ -amanitin-sensitive genes expressed during the 1- and 2-cell stages, and we used Expression Analysis Systematic Explorer (EASE) and Ingenuity Pathway Analysis (IPA) to identify biological and molecular processes represented by these genes, as well as interactions among them. We find that although the 1-cell embryo is transcriptionally active, we did not detect any transcripts present on the MOE430 GeneChip set to be  $\alpha$ -amanitin-sensitive. Thus, what the BrUTP incorporation represents remains elusive. About 17% of genes expressed in the 2-cell embryo are  $\alpha$ -amanitin-sensitive. EASE analysis reveals that genes involved in ribosome biogenesis and assembly, protein synthesis, RNA metabolism and transcription are over-represented, suggesting that genome activation during 2-cell stage may not be as global and promiscuous as previously proposed. IPA implicated *Myc* and *Hdac1* as candidate genes involved in genome activation and the development of the transcriptionally repressive state, respectively.

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## Introduction

Zygotic gene activation (ZGA) is the first major developmental event that occurs following fertilization (Schultz, 2002). Meiotic maturation initiates degradation of most maternal mRNAs and is essentially complete by the late 2-cell stage in mouse. For oocyte-specific transcripts, such as *Hlfoo* (Tanaka et al., 2001) and *Msy2* (Yu et al., 2001) that are not re-expressed later in development, destruction of these maternal mRNAs restricts the length of time that these genes can function. For transcripts that are common to both the oocyte and embryo, for example, actin, ZGA is essential for replacing the degraded maternal transcripts with zygotic transcripts. ZGA is also responsible

for the dramatic reprogramming of gene expression that occurs during the 2-cell stage in mouse and likely drives the conversion of the highly differentiated oocyte into totipotent 2-cell blastomeres. ZGA is essential for development because 2-cell embryos do not cleave when cultured in the presence of  $\alpha$ -amanitin, which inhibits RNA polymerase II activity.

Although genome activation is clearly evident in 2-cell embryos, transcription initiates during the 1-cell stage as assessed by either BrUTP incorporation (Aoki et al., 1997; Bouniol et al., 1995) or expression of a plasmid-borne reporter gene (Ram and Schultz, 1993). In both cases, the male pronucleus (PN) supports a higher level of transcription than the female PN (Aoki et al., 1997; Wiekowski et al., 1993). Moreover, the level of transcription is significant in the late 1-cell embryo, reaching 30–40% that observed in the late 2-cell embryo (Aoki et al., 1997).

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Nevertheless, expression of only MuERV-L (a repetitive element and as such may not be reflective of single-copy genes) in 1-cell embryos has been reported to date (Hamatani et al., 2004). Expression of a  $\beta$ -actin promoter-driven luciferase transgene has also been observed in 1-cell embryos (Kigami et al., 2003). Because the absolute level of transcript abundance was not quantified in these reports, it has not been established that their expression represents mature transcripts capable of being translated.

Transcription and translation may be uncoupled in the 1-cell embryo (Nothias et al., 1996). Consistent with this proposal is that luciferase activity is detected several hours after expression of the luciferase transgene (Matsumoto et al., 1994), that is, the transcript is detected in 1-cell embryos but the protein is first detected in 2-cell embryos. Thus, the ability of the 1-cell embryo to generate functional transcripts, that is, transcripts that are spliced, capped, polyadenylated, transported to the cytoplasm, and translated is unresolved.

Superimposed on genome activation is the development of a chromatin-based transcriptionally repressive state during the 2-cell stage and the requirement for an enhancer for efficient gene expression of a plasmid-borne reporter gene (Aoki et al., 1997; Davis et al., 1996; Henery et al., 1995; Ma et al., 2001; Majumder et al., 1993). This repression and enhancer requirement are relieved by inducing histone acetylation or by inhibiting the second round of DNA replication. Chromatin remodeling that occurs during the 1- and 2-cell stages has been proposed to provide a window of opportunity for transcription factors to gain access to their *cis*-cognate DNA-binding sequences (Ma et al., 2001). Thus, any gene whose promoter is accessible and for which the relevant transcription factors exist would be transcribed. This may account, at least in part, for the high incidence of transcripts derived from repetitive elements in the 2-cell embryo (Evsikov et al., 2004; Svoboda et al., 2004). A function of the transcriptionally repressive state would be to repress transcription of such opportunistically expressed genes and sculpt an expression profile compatible with development in which genes that use a strong promoter or enhancer are expressed. In addition, development is accompanied by a more efficient use of TATA-less promoters (Davis and Schultz, 2000; Majumder and DePamphilis, 1994), which may also play a role in establishing the appropriate pattern of gene expression required for development.

Recent studies using microarrays, however, suggest that genome activation may not be as promiscuous as previously envisioned (Hamatani et al., 2004; Wang et al., 2004; Zeng et al., 2004), but rather far more selective with genes involved in transcription and RNA processing being preferentially expressed (Zeng et al., 2004). What remains elusive is identifying the genes that are transcribed at the onset of ZGA—perhaps genes critical for the sustaining ZGA—and the development of the transcriptionally repressive state. We report here the transcript profiles in 1-cell and 2-cell mouse embryos treated with  $\alpha$ -amanitin to identify genes

that are activated during the course of ZGA. We then used Expression Analysis Systematic Explorer (EASE) analysis and Ingenuity Pathway Analysis to identify major biological themes that occur during ZGA and to predict key networks of genes that function during this developmental transition.

## Materials and methods

### *Embryo collection and culture*

One-cell mouse embryos were collected from superovulated female CF-1 mice (Harlan) mated to B6D2F1/J males (Jackson Laboratory) as previously described (Aoki et al., 1997). The embryos were cultured in KSOM plus amino acids (abbreviated as KSOM) in an atmosphere of 5% O<sub>2</sub>/5% CO<sub>2</sub>/90% N<sub>2</sub> at 37°C (Erbach et al., 1994; Ho et al., 1995). For the metabolic labeling and splicing study, embryos were synchronized by culling them as previously described (Ram and Schultz, 1993). To block RNA polymerase II-dependent transcription,  $\alpha$ -amanitin (Sigma-Aldrich) was added to the culture medium at a final concentration of 24  $\mu$ g/ml at the time points indicated in the text and Fig. 2.

For microarray studies, unfertilized eggs were collected from superovulated female CF-1 mice at 14–16 h post-hCG as previously described (Endo et al., 1987). One-cell embryos were collected (see above) at 14–16 h post-hCG and cultured in KSOM with or without  $\alpha$ -amanitin prior to PN formation to insure that transcription was inhibited; fertilization occurs ~12 h post-hCG, and PN formation around 5–7 h post-fertilization (Moore et al., 1996). Embryos were then recovered from the culture dishes at 24 h (1-cell) and 44 h post-hCG (2-cell), which corresponds to S in the 1-cell and G2 in the 2-cell, respectively. Four pools of embryos (~325 eggs, 335 1-cell and 380 2-cell per pool) from each stage/treatment were collected from separate sets of fertilized mice. The samples were then processed for microarray analysis as described below.

### *[<sup>35</sup>S] methionine metabolic labeling of mouse embryos*

Synchronized 2-cell embryos obtained by culling were metabolically radiolabeled in KSOM without amino acids but containing 1 mCi/ml [<sup>35</sup>S]methionine (1500 Ci/mmol, Amersham) for 3 h from 42 to 45 h post-hCG (which corresponds to G2) in the presence or absence of  $\alpha$ -amanitin. The radiolabeled samples were then subjected to SDS-PAGE (10% gel); samples with equal numbers of acid-insoluble cpm were applied to each lane. Radiolabeled proteins were detected using a phosphorimager.

### *Microinjection of 1-cell embryos*

One-cell embryos (collected at 16–18 h post-hCG) that developed a PN within a 30-min period were culled and

incubated in KSOM. The male PN of 1 cell embryos was microinjected 4 h after PN formation (early S phase), and either one or both nuclei of 2 cell embryos was injected 19–20 h (early S phase) or 24 h (late S phase) after PN formation. The PN or nuclei were injected with 2–10 pl of a 10-ng/ $\mu$ l solution of a pGL2-Control plasmid (Promega) in 10 mM Tris-HCl, pH 7.6, containing 0.1 mM EDTA as previously described (Ram and Schultz, 1993). Approximately 3–15,000 copies of the plasmid were injected. Control embryos were injected with 5 pl of H<sub>2</sub>O. The microinjected embryos were then transferred to and cultured in KSOM; ~75% of the injected embryos routinely survived the injection procedure. Embryos were then recovered at late G2 of the 1-cell or 2-cell stage (13 h and 40 h after injection, respectively) for subsequent RNA extraction and RT-PCR analysis.

#### *RNA extraction and RT-PCR*

Total RNA was extracted from embryos in 150  $\mu$ l of Trizol (Invitrogen) containing 20  $\mu$ g of rRNA or glycogen as carrier. RT-PCR for the splicing study was performed as previously described (Zeng and Schultz, 2003), except nested PCR was performed. The primers used were A [5'-GCCTGGTGCTACGCCTGAATAA-3'], D [5'-CCA-CCACTGCTCCCATTCATCA-3'] and E [5'-GTCAGC-AGTAGCCTCATCATC-3']. Ten embryo equivalents of template RNA was used for the first PCR with primers A and E for 20 cycles (95°C for 10 s and 60°C for 15 s), 1/10 of which was subjected to a second nested PCR with primers A and D for 32 cycles (95°C for 15 s, 58°C for 30 s, and 72°C for 1 min). PCR products were visualized on a 3% (3:1) NuSieve Agrose gel, followed by gel extraction of the specific fragments (Qiagen) for sequencing analysis.

Real-time RT-PCR analysis was performed with the ABI TaqMan Assay-on-Demand probe/primer set (Zeng et al., 2004) Mm00487803\_m1 (*Myc*), as well as the ABI TaqMan Assay-by-Design probe/primer sets for MuERV-L and *Hdac1*. Two embryo equivalents of template RNA were used for each real-time PCR reaction with a minimum of three replicates (from independently collected embryos per replicate) as well as a minus RT and minus template controls for each gene. Quantification was normalized to exogenously added GFP mRNA that was added in equal amounts per embryo equivalent during RNA extraction (Anger et al., 2005), using the comparative CT method (ABI PRISM 7700 Sequence Detection System, user bulletin #2).

#### *Microarray analysis*

Total RNA from 4 replicates of each embryo stage/treatment was used for linear, two-round amplification by in vitro transcription and target cRNA preparation according to the Affymetrix Small Sample Prep Technical Bulletin ([www.affymetrix.com](http://www.affymetrix.com)). Fifteen  $\mu$ g per replicate of fragmented cRNA samples were serially hybridized to

MOE430A and MOE430B GeneChips, then processed according to the manufacturer's instructions (GeneChip Analysis Technical Manual, [www.affymetrix.com](http://www.affymetrix.com)) at the Penn Microarray Facility.

Microarray analysis was performed as described in detail in Zeng et al. (2004), and the resulting data from this and our previous study are available at the Gene Expression Omnibus ([www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo)). Briefly, Microarray Analysis Suite 5.0 (MAS, Affymetrix) was first used to quantify microarray signals with default analysis parameters and global scaling to target mean = 150. The MAS metrics output was then loaded into GeneSpring v6 (Silicon Genetics) with per chip normalization to the 50th percentile and per-gene normalization to the median. A non-redundant, filtered list was created of all genes detected (MAS "P" call) in at least three of four replicates in at least one embryo stage/treatment (called "all genes detected" below). K-mean hierarchical clustering with this gene list, as well as all genes on the MOE430 chip set, was used to construct clustering dendrograms to visualize inter-relationships between different embryo samples. The MAS metrics output of all genes on the GeneChip set after GeneSpring normalization was also used for a one-way ANOVA analysis for microarrays (default parameters with a FDR <5%), as well as Principle Component Analysis (PCA) (default parameters except 2-fold change threshold for clusters) using the NIA Array Analysis Tool (<http://lgsun.grc.nia.nih.gov/ANOVA/index.html>).

The GeneSpring "filter on fold change", "filter on flags", and "draw gene" tools were applied to the statistically significant candidate lists to locate genes that showed different characteristics and patterns (e.g., genes that are  $\alpha$ -amanitin-sensitive in the 2-cell cultured embryo and also present with at least 5-fold higher expression in in vivo developed 2-cell compared to 1-cell). For simplicity, the following abbreviations are used throughout: 1CC or 2CC refer to 1-cell or 2-cell embryos cultured in KSOM; 1CA or 2CA refer to 1-cell or 2-cell embryos cultured in KSOM +  $\alpha$ -amanitin; 1C and 2C refer to 1-cell or 2-cell embryos developed in vivo with minimal manipulation in vitro. Note that MAS output data from our previous microarray studies of in vivo developed 1- and 2-cell embryos were included for GeneSpring analyses, as well as used for ANOVA analysis to generate expression profiles of genes in different conditions/treatment. We used in vitro samples only as the first filter and to identify candidate genes that are  $\alpha$ -amanitin-sensitive, and to place these candidates in a biological context, we also compared the in vivo sample set in eggs, 1-cell and 2-cell embryos. At no time were in vivo and in vitro samples directly compared to each other and used to generate statistically significant gene lists. Lists of candidate genes generated from these pattern searches were then imported to EASE v2.0 to test for overrepresentation of biological processes in each subset list (Hosack et al., 2003). EASE analysis with Bonferroni multiplicity correction tested each subset list against their corresponding population lists (e.g., all non-redundant genes detected), and an EASE

score was calculated for likelihood of overrepresentation in the Gene Ontology Consortium annotation categories GO biological process, GO cell component and GO molecular function, as well as KEGG pathways and SwissProt keywords.

Ingenuity Pathways Analysis (IPA) version 2.0 was used to search for possible biological pathways and the inter-relationships between network genes in the subsets of candidate genes with particularly interesting patterns. A detailed description of IPA can be found on [www.ingenuity.com](http://www.ingenuity.com). Data sets containing the Affymetrix gene identifiers and their corresponding expression fold-change values, and *P* values from ANOVA analysis were uploaded as tab-delimited text files. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. A fold-change cutoff of at least 1.4 was set between stage/treatment to filter further the genes whose expression was significantly differentially regulated. These genes, called Focus Genes, were then used as the starting point for generating biological networks.

To start building networks, the program queries the Ingenuity Pathways Knowledge Base for interactions between Focus Genes and all other gene objects stored in the knowledge base, and generates a set of networks. IPA then computes a score for each network according to the fit of the network to the set of focus genes. The score is the negative log of a *P* value and indicates the likelihood of the Focus Genes in a network being found together due to random chance. A score of 2 indicates that there is a 1 in 100 chance that the Focus Genes are together in a network due to chance, a score of 3 corresponds to 1 in 1000, and so forth. Therefore, scores of 2 or higher represent a 99% confidence level. Biological functions are then calculated and assigned to each network.

It should be noted that a recent study reported that many transcripts expressed in oocytes (and hence 1-cell embryo) and 2-cell embryos are chimeric in which an LTR class III retrotransposon sequence is located at the 5' end (Peaston et al., 2004); the function of these transcripts in preimplantation development remains unresolved. The microarrays used here do not discriminate between these transcripts and their bona fide counterparts because the array probes used are biased towards the 3' end of the transcript.

### Cross-platform analysis

The mapping between the Affymetrix MOE430 GeneChip set and the NIA 22K oligomer microarray platforms were determined by using BLASTN with default parameters to compare each NIA oligomer to a database of MOE430 consensus sequences (downloaded from NetAffx). Blast matches greater than or equal to 55 bp in length and greater than or equal to 95% identity were considered to represent the same gene. This mapping was then used as input to a Perl script that compares the  $\alpha$ -amanitin-sensitive genes between this study and the combined, non-redundant  $\alpha$ -

amanitin-sensitive genes from all time points from the Hamatani study (Hamatani et al., 2004) to identify the overlapping set (or concordance) between the two studies.

## Results and discussion

### *Hierarchical cluster analysis and identification of $\alpha$ -amanitin-sensitive gene sets*

We previously determined that following fertilization there are increases in relative transcript abundance of many transcripts in 1-cell embryos (Zeng et al., 2004). Many of these changes are likely due to recruitment of maternal mRNAs that entails polyadenylation and would result in more efficient dT priming, and hence an apparent increase in relative abundance. Nevertheless, given that the 1-cell embryo is transcriptionally active, it is also possible that some of these increases are due to transcription. In addition, results of numerous studies indicate that transcription is responsible for many of the changes in gene expression that occur during the 2-cell stage (e.g., Latham et al., 1991). To identify these newly expressed genes, we analyzed the transcript profiles in 1- and 2-cell embryos that had been cultured in the presence of  $\alpha$ -amanitin. Because culture can influence the pattern of gene expression (Christians et al., 1995; Rinaudo and Schultz, 2004), we included microarray data sets of 1-cell and 2-cell embryos that developed in vivo from our previous study (Zeng et al., 2004) in the analysis presented here.

An unsupervised hierarchical clustering using either all the transcripts on the MOE430 chip set or the non-redundant list of transcripts that are expressed in the early preimplantation embryos (Table 1) was constructed using microarray data from in vitro treated embryos and 1- and 2-cell embryos

Table 1  
Number of genes detected in in vivo developed and in vitro treated 1-cell and 2-cell embryos

Condition	Genes detected	$\alpha$ -amanitin-sensitive	$\alpha$ -amanitin-sensitive and detected
1-cell in vivo	9340	–	0
1-cell culture	10422	1	0
1-cell + $\alpha$ -am	10262	–	–
2-cell in vivo	10579	–	1464
2-cell culture	10597	2607	1819
2-cell + $\alpha$ -am	8906	–	–
Total	13679		

The number of genes detected (Affymetrix “P” call in at least 3 of 4 replicates) on the MOE430A and B GeneChips in each stage/treatment, as well as the total number of non-redundant genes detected from all six conditions, are listed in the second column. One-way ANOVA was used to identify genes showing a statistically significant expression difference between  $\alpha$ -amanitin-treated and untreated 1- and 2-cell embryos and the resulting number of genes is listed in the third column. This  $\alpha$ -amanitin-sensitive gene list is then filtered against the list of genes that are detected in 1- and 2-cell embryos and the subset is listed in the fourth column. See Supplementary Table S1 for the list of  $\alpha$ -amanitin-sensitive genes.

that developed in vivo (Fig. 1A). As anticipated, the 1- and 2-cell embryos clustered separately. The sub-clustering of 2-cell embryos that developed in vitro or in vivo likely represents the effect of culture on gene expression and that

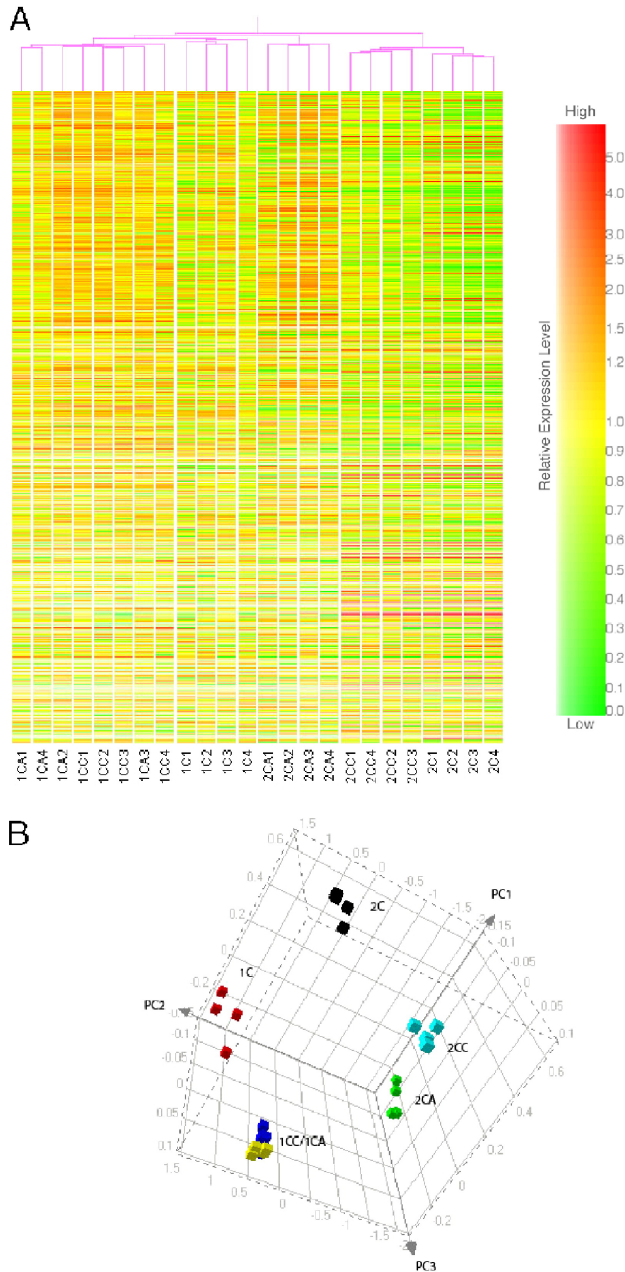


Fig. 1. Hierarchical clustering and PCA analysis. (A) Hierarchical clustering analysis of all samples from different developmental stages or treatments. Unsupervised clustering in GeneSpring was used to analyze similarities among replicate samples across all stages tested. Replicate samples are indicated at the bottom of the figure. Colors correspond to relative RNA abundance for the 13,679 transcripts (Unigene accessions) detected (Affymetrix “Present” call), each of which is represented by a horizontal bar. 1CC and 2CC refer to 1-cell and 2-cell embryos cultured in KSOM; 1CA and 2CA refer to 1-cell or 2-cell embryos cultured in KSOM containing  $\alpha$ -amanitin; 1C and 2C refer to 1-cell and 2-cell embryos that developed in vivo. (B) Principal component analysis of gene expression of all groups as in the hierarchical clustering analysis.

development in vitro lags development in vivo. For example, the increase in *Hsp70.1* expression that occurs between the 1- and 2-cell stages is much greater when the 1-cell embryos are cultured (Christians et al., 1995). Also anticipated is that 2-cell embryos cultured in the presence of  $\alpha$ -amanitin (2CA) clustered with the 1-cell rather than the 2-cell embryos because the transcripts present in these treated 2-cell embryos are mainly of maternal origin.

One-cell embryos that developed in vivo clustered separately from those that developed in vitro in either the presence or absence of  $\alpha$ -amanitin. As with 2-cell embryos, this likely was due to processes such as maternal mRNA degradation occurring with different kinetics than in embryos developing in vivo. Of note, however, was that the replicates of 1-cell cultured embryos did not cluster according to the presence or absence of  $\alpha$ -amanitin treatment, but rather clustered into one large tree branch. Thus, all the replicates of these 1-cell embryos, regardless of  $\alpha$ -amanitin treatment, showed very similar gene expression patterns. This implies that although the 1-cell embryo is transcriptionally active, few poly (A)-containing transcripts are generated. The failure to detect  $\alpha$ -amanitin-sensitive transcripts cannot be attributed to the ineffectiveness of  $\alpha$ -amanitin to inhibit transcription in 1-cell embryos. Real-time PCR analysis of MuERV-L, which is transcribed in the 1-cell stage (Hamatani et al., 2004; Kigami et al., 2003), showed that a 4.5-fold increase occurs between the egg and 1-cell stage, and in the presence of  $\alpha$ -amanitin this increase is 1.6-fold, that is, a 65% decrease in level of expression in the  $\alpha$ -amanitin-treated embryos. Thus, the cluster analysis suggests little de novo transcription from the zygotic genome in 1-cell embryos.

Principle Component Analysis (PCA) confirmed the relationships between the samples from different treatments and stages of the embryos (Fig. 1B). The 1CC/1CA and 2CA resided closer to 1C in the PC2/PC3 plane, the 2CC and 2C were closer to each other and towards the opposite PC2/PC3 plane, and the 1C and 2C were grouped against PC1/PC2 plane with respect to other groups. Thus, the concordance of the hierarchical cluster analysis and the PCA analysis with previous results collected by totally independent methods provides confidence in conclusions drawn from these microarray data sets. (See below for further discussion on the concordance of the two studies.)

One-way ANOVA analysis using Affymetrix MAS output data after GeneSpring normalization was used to search for the  $\alpha$ -amanitin-sensitive candidate genes that are statistically different between  $\alpha$ -amanitin-treated vs. control embryos. Table 1 lists the number of transcripts that are detected (using GeneSpring filter on “Flag” according to the Affymetrix “P” call) on the MOE430 GeneChip set and the number of  $\alpha$ -amanitin-sensitive transcripts in 1- and 2-cell embryos. In contrast to the expected large number of  $\alpha$ -amanitin-sensitive transcripts detected in the 2-cell (discussed below) and consistent with the cluster analysis, no  $\alpha$ -amanitin-sensitive transcripts were detected in the

1-cell embryo (Table 1). Hamatani et al. (2004) using a different platform of long oligomer arrays derived from cDNA libraries to analyze expression profiles of  $\alpha$ -amanitin-treated embryos only detected 1 de novo transcript in 1-cell embryos, MuERV-L (M. Ko, personal communication). Thus, recruitment of maternal mRNAs is likely responsible for our detecting  $\sim 1500$  transcripts that are expressed higher in the 1-cell embryo when compared to other stages of preimplantation development (Zeng et al., 2004).

#### TRC expression in the 1-cell embryo

Given that the extent of BrUTP incorporation by 1-cell embryos is  $\sim 30$ – $40\%$  that of a 2-cell embryo (Aoki et al., 1997), the failure to detect a family of  $\alpha$ -amanitin-sensitive transcripts in the 1-cell embryo using microarrays highlights the conundrum as to what the transcription in the 1-cell embryo represents, for example, are bona fide transcripts

actually made? As an alternative approach, we examined if transcripts for the transcription-requiring complex (TRC) are expressed in the 1-cell embryo. The TRC, which is  $\alpha$ -amanitin-sensitive, is an accepted marker for ZGA and encodes a family of structurally-related proteins of  $M_r = 70,000$  (Conover et al., 1991). Its identity, however, is not known, and hence the TRC might not be properly annotated or present on the Affymetrix MOE430 GeneChip. TRC expression, which can be detected as early as 2–3 h following cleavage to the 2-cell stage, constitutes 4–6% of total protein synthesis in 2-cell embryos (Conover et al., 1991) and hence its transcripts are likely expressed at reasonably high levels.

Because the TRC protein synthesis is rapidly detected following cleavage to the 2-cell stage, we examined whether this synthesis results from translation of TRC transcripts expressed in the 1-cell embryo but not yet translated due to the uncoupling of transcription and translation (Nothias et al., 1996) (Fig. 2). One-cell embryos were cultured in the

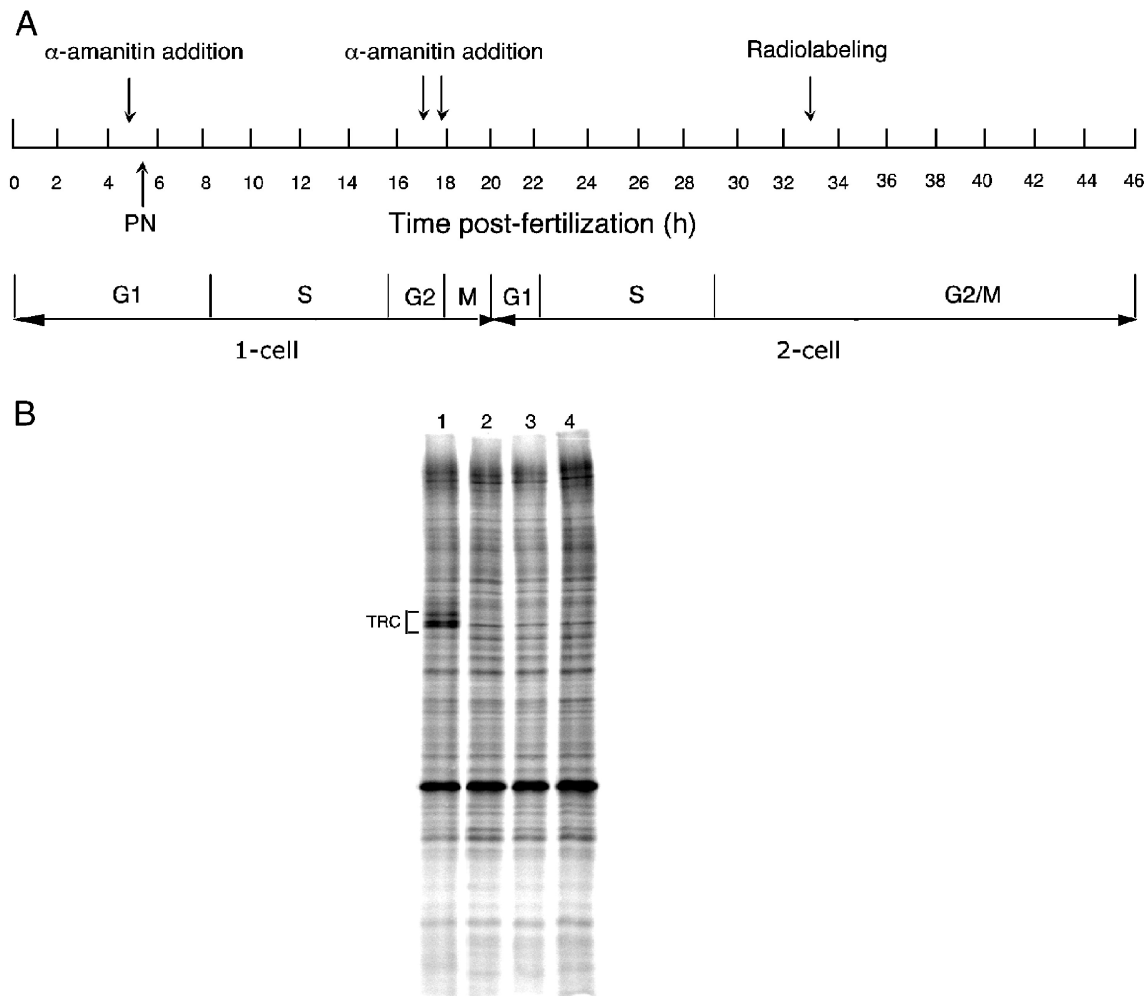


Fig. 2. TRC expression in 1-cell embryos. (A) Schematic of the time-course of the experimental treatment. The time line of the first and second cell cycles was adapted from Moore et al. (1996). PN, pronucleus formation. (B) TRC expression in metabolically radiolabeled embryos. All embryos were radiolabeled at the mid 2-cell stage (45 h post-hCG). Lane 1, control embryos; lanes 2–4, 1-cell embryos cultured in KSOM containing  $\alpha$ -amanitin starting at 5, 17, and 18 h post-fertilization (G2/M of the 1-cell stage). The position of the TRC is indicated by the bracket. The experiment was performed three times and similar results were obtained in each case; shown is a representative example.

KSOM until the late G2 stage of the 1-cell (30–31 h post-hCG) at which time they were transferred to KSOM containing  $\alpha$ -amanitin. Following cleavage to the 2-cell stage, the embryos were assessed for TRC synthesis.

Following addition of  $\alpha$ -amanitin to 1-cell embryos at a time corresponding to the late G2 stage of the 1-cell embryo, no TRC synthesis was observed in the 2-cell embryos, as was also the case with embryos cultured in  $\alpha$ -amanitin from the early 1-cell stage. In contrast, TRC expression was readily observed in the untreated control 2-cell embryos. Thus, it is unlikely that functional TRC transcripts—transcripts that are expressed, processed, transported to the cytoplasm and translated—are expressed in the 1-cell embryo. We cannot exclude, however, that TRC transcripts were degraded by the time of metabolic radiolabeling.

#### *Splicing competence in the 1-cell embryo*

Our inability to detect a set of  $\alpha$ -amanitin-sensitive transcripts in 1-cell embryos could be due to deficiencies in transcription and processing, export, and mRNA stability. It is unlikely that the newly synthesized transcripts are so rapidly degraded as not to be detected. Although mRNA injected into 1-cell embryos can be degraded ~65-fold faster than mRNA injected into oocytes (Ebert et al., 1984), the detection of TRC synthesis in G2 of the 1-cell embryo following transplantation of a 2-cell nucleus to an enucleated 1-cell embryo (Latham et al., 1992) suggests rapid degradation of nascent transcript is less likely the case in 1-cell embryo. Furthermore, this result also suggests that the late 1-cell embryos can support transcription once ZGA initiates. Thus, a more likely cause for the lack of functional transcripts in 1-cell embryos are defects or inefficiencies in transcription, mRNA processing or mRNA export.

An RNA transcript undergoes four major interlinked processing events—capping, splicing, cleavage, and polyadenylation—before it becomes a translatable mRNA that is exported from the nucleus (Lee and Young, 2000). Moreover, these processes are directly associated with RNA polymerase II (Pol II) at all three stages of transcription, that is, initiation, elongation, and termination. Deficiencies in one or more of these events could produce transcripts lacking a poly(A) tail. Such transcripts would not be amplified by our protocol and hence could account for our failure to detect expression of  $\alpha$ -amanitin-sensitive 1-cell transcripts. Although MuERV-L is expressed in the 1-cell embryo, it is intronless, and hence splicing is not required for generating functional transcripts. MuERV-L is also a multicopy element and its transcriptional regulation may not reflect that of single-copy genes. Plasmid-borne reporter genes (Ram and Schultz, 1993) or transgenes (Matsumoto et al., 1994) expressed in 1-cell embryos have introns, but splicing was not evaluated in the 1-cell embryo. Thus, we examined if deficiencies in splicing could serve as the molecular basis for our failure to detect  $\alpha$ -amanitin-sensitive transcripts in 1-cell embryos.

A plasmid-borne reporter gene (pGL2-Control plasmid) containing the SV40 small intron in the 3'UTR (Fig. 3A) was microinjected into the male PN of 1-cell embryos at G1/early S phase. The spliced products (172 bp and 243 bp) were detected in 1-cell embryos in G2 by RT-PCR using specific primer pairs that flank the intron (Fig. 3B, lane 1). The spliced products were also detected when the plasmid was injected in the 2-cell early S phase and assayed at the 2-cell late G2 stage (Fig. 3B, lane 3), but not if assayed at the late S/early G2 (data not shown). DNA sequencing analysis confirmed the identity of the 172 bp and 243 bp fragments obtained from 1-cell embryos. It is therefore unlikely that failure to detect  $\alpha$ -amanitin-sensitive transcripts in 1-cell embryos is due to a deficiency in splicing.

One-cell embryos possess functional RNA polymerase II (Latham et al., 1992), in which a significant fraction of the hyperphosphorylated carboxy-terminal domain of the largest subunit observed in the egg is dephosphorylated 22 h post-hCG (Bellier et al., 1997). However, deficiencies in some core components of the transcription machinery or various elongation/cleavage factors could result in either premature termination or failure to terminate. In either case, this could account for the significant amount of BrUTP incorporation that is observed and the failure to generate poly(A)-containing transcripts that would be amplified. Clearly, deficiencies in transcription and processing, including initiation, elongation, and termination, need to be examined further. Of note is that our microarray data reveal that among genes that are  $\alpha$ -amanitin-sensitive in vitro, those related to RNA transcription elongation (*Ercc3*, *Gtf2f1*), RNA processing (*Prpf4b*, *Cstf1*), splicing (*Ddx20*, *Gemin5*, *Sip1*, *Snrpd2*), and polyadenylation (*Cpsf5*) also increase between the 1- and 2-cell stages in vivo.

RNA polymerase II is also responsible for transcription of several snRNAs (Henry et al., 1998), which are involved in splicing, and snoRNAs (Gerbi, 1995), which are involved in pre-ribosomal RNA processing and post-transcriptional modification. We are currently ascertaining if the observed BrUTP incorporation in 1-cell embryos is due to expression of these RNAs that are not polyadenylated. If so, enhanced expression of snRNAs and snoRNAs, coupled with up-regulation of genes involved in producing functional transcripts (see above), may serve to prepare the embryo for the major onset of ZGA in the 2-cell embryo.

#### *Identification of biological themes from over-represented annotation categories for 2-cell nascent transcripts*

In contrast to the 1-cell embryo, the major period of ZGA and reprogramming of gene expression unequivocally occurs during the 2-cell stage, and microarray analyses have highlighted the extent of this reprogramming (Hamatani et al., 2004; Wang et al., 2004; Zeng et al., 2004). These studies demonstrated robust and dynamic patterns of gene expression during early mouse develop-

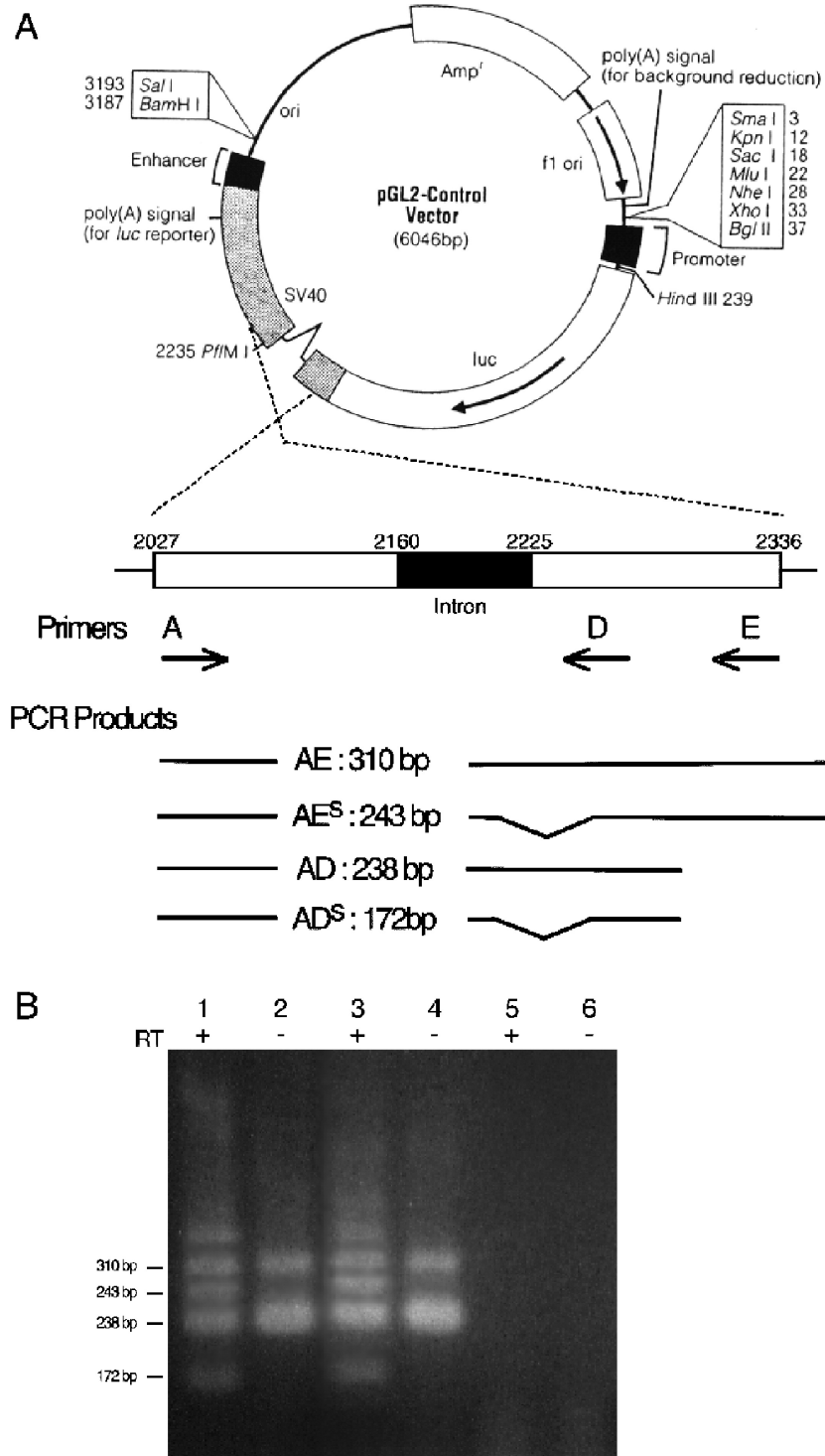


Fig. 3. Splicing competence of 1-cell embryos. (A) pGL2-Control plasmid and experimental design to detect splicing by nested PCR. The plasmid map was adapted from Promega technical manual. (B) Detection of splicing products in 1-cell or 2-cell embryos microinjected with the pGL2-Control plasmid. Lanes 1 and 2, 1-cell embryo injected during G1/early S phase and detected in late G2; lanes 3 and 4, 2-cell embryo injected during G1/early S phase and detected in late G2; lanes 5 and 6, 1-cell control embryo injected with H<sub>2</sub>O and assayed at the same time point as in lanes 1 and 2. RT, + or – refer to samples that were either reversed transcribed (+) or not (–). The experiment was performed twice and similar results were obtained in each instance. The presence of bands in the –RT sample are due to residual plasmid DNA that survived the DNase I treatment.

ment, identified large numbers of genes, and predicted relevant biological programs/themes and signal pathways in each stage of development including the 2-cell stage.

We extended our previous microarray analysis on transcript profiles in preimplantation embryos by identifying  $\alpha$ -amanitin-sensitive genes in 2-cell embryos (Table 1).



Transcripts represented by 2607 Unigenes accessions are  $\alpha$ -amanitin-sensitive, of which 1819 are called detected by Affymetrix MAS “present” call and constitute 17% of all genes detected in the 2-cell embryo. Because we often observe that useful information can be drawn from expression profiles (and confirmed by quantitative RT-PCR) even with genes that have an “absent” call by the MAS program, we decided to include all genes that were significantly  $\alpha$ -amanitin-sensitive as initial candidate genes for further analysis with more stringent filters.

Of the transcripts up-regulated between the 1- and 2-cell stages, 60% that increased by at least a statistically significant 1.4-fold were  $\alpha$ -amanitin-sensitive, whereas 86% of the genes up-regulated by at least 5-fold were  $\alpha$ -amanitin-sensitive (Table 2). A similar number of  $\alpha$ -amanitin-sensitive transcripts were also found in the subset of genes that were up-regulated in in vivo developed 2-cell embryos. The number of  $\alpha$ -amanitin-sensitive transcripts we observed is higher than that reported in another study (Hamatani et al., 2004). This may reflect different microarray platforms, different sets of genes on the arrays, different culture conditions, and different statistical analyses used in the two studies.

Expression profiles of a few genes known to be  $\alpha$ -amanitin-sensitive and up-regulated at the 2-cell stage were also confirmed in our analysis; these genes include *Hsp70.1*, *Eif1a*, *U2af1-rs1*, and *Rpl23*. The Expression Analysis Systematic Explorer (EASE) program was then used to identify annotation categories over-represented among genes that were  $\alpha$ -amanitin-sensitive in 2-cell embryos. EASE analysis implements a statistical measure to discover biological themes in a particular list of genes of interest regardless of the expression levels of the genes. Lists of genes that showed  $\alpha$ -amanitin sensitivity with the MAS detection filter were analyzed against all genes detected in 2-cell embryos, whereas genes that showed  $\alpha$ -amanitin sensitivity without the MAS detection filter were analyzed against all genes represented on the MOE430 GeneChip set. Interestingly, the two predominate functional categories over-represented in the  $\alpha$ -amanitin-sensitive genes sets are related to protein biosynthesis, and RNA transcription and processing (Table 3, see Supplementary Table S2 for a complete list of genes in each functional category). In addition, cell cycle genes were also over-represented in the 2-cell when comparing  $\alpha$ -amanitin-sensitive genes to all genes on the

Table 2  
Number of  $\alpha$ -amanitin-sensitive genes in in vitro developed 2-cell embryos

Fold-Change (2CC/1CC)	1.4×	2×	3×	5×
2CC>1CC	2990	2944	2453	1482
2CC>1CC and $\alpha$ -amanitin-sensitive	2033	2017	1815	1282
% $\alpha$ -amanitin-sensitive	68.0	68.5	74.0	86.5

Number of Affymetrix probe sets on the MOE430 GeneChip set that are statistically significantly changed by at least the fold-change indicated in the column header.

Table 3

Selected processes over-represented in  $\alpha$ -amanitin-sensitive 2-cell transcripts

Over-represented categories	EASE score	No. of genes
Ribosome biogenesis and assembly	2.84E-14	36
rRNA processing	1.65E-02	9
Biosynthesis—protein biosynthesis	2.00E-12	101
(A) Protein metabolism	2.42E-04	234
Protein folding	1.95E-03	19
(B) Translation—translational initiation	1.14E-02	13
Nucleic acid metabolism	1.61E-07	264
Nucleoside/ribonucleoside monophosphate biosynthesis	1.39E-02	6
RNA metabolism-RNA processing	7.75E-12	67
mRNA processing	2.48E-06	37
(1) RNA splicing	5.17E-04	25
(2) mRNA splicing	1.04E-03	23
Transcription	3.72E-04	165
(A) Transcription, DNA-dependent	1.05E-02	147
(B) Transcription from Pol I promoter	2.82E-02	10

The EASE analysis tool was used to calculate likelihood of over-representation for annotation categories associated with  $\alpha$ -amanitin sensitive 2-cell transcripts. Listed in the table are EASE characterized biological processes for annotated genes in the  $\alpha$ -amanitin sensitive set of genes that are detected on the MOE430 chips, with a score less than 0.05. The number of genes in each category is shown next to the EASE score. The genes listed in each functional category can be found in Supplemental Table S2.

MOE430 GeneChip and may underlie the shift from a meiotic, non-proliferative phase to mitotic, proliferative phase.

We previously reported that transcription and RNA processing, metabolism and cell cycle genes are over-represented among the 2-cell up-regulated genes (“2-cell transient”), compared to the other stages examined (Zeng et al., 2004); Hamatani et al. (2004) also noted enhanced zygotic expression of genes involved in RNA processing. The results reported here demonstrate that these functions are largely due to genes transcribed by the 2-cell embryo. More important, however, is that the results of this and our previous study suggest that genome activation is not as global and promiscuous as we previously proposed (Ma et al., 2001). The basis for this proposal was that the extensive chromatin remodeling that occurs during the first two cell cycles provides a window of opportunity for expression of genes not normally expressed. The function of the chromatin-mediated transcriptionally repressive state that develops in the 2-cell embryo is to extinguish expression of these genes that are opportunistically expressed as a consequence of ZGA. In fact, our microarray studies indicate that a large number of genes are transiently expressed (Zeng et al., 2004). Nevertheless, our EASE analyses suggest that subsets of genes with particular functions, for example, transcription and RNA processing, are selectively expressed during ZGA. Expression of such genes may provide a positive feedback mechanism to ensure that ZGA is irreversible.

Table 4

Top 10 networks generated from IPA for  $\alpha$ -amanitin-sensitive transcripts in the 2-cell cultured embryos

Network ID	Genes in network	Score	# Focus genes	Top categories
1	<i>Atpi, Bat1, Cpd, Ddx18, Ddx21, Gdi2, Gm2a, H2afz, Imp-1, Jtv1, Lamp2, Mina53, Myc, Mycn, Ndr1, Nol5a, Pls3, Rpl9, Rpl19, Rpl21, Rpl23, Rpl27, Rpl30, Rpl32, Rpl35, Rpl41, Rps13, Rps19, Rps20, Rps23, Rps27, Rps4x, Sardh, Shmt1, Tde1</i>	32	35	Cell cycle, protein synthesis, cancer
2	<i>Apex1, Bag4, Ccnd1, Ccne1, Ccne2, Cdkn1a, Cul3, Dnaj1, Foxg1b, Hdac1, Hes1, Hesx1, Hmgb1, Hmgb2, Hspa8, Hsph1, Jarid1b, Klf4, Mta2, Nme2, Pax9, Pim1, Prkch, Procr, Prtn3, Rad9a, Rnf7, Set, Skp2, Terf1, Tle1, Tob1, Topbp1, Vhl, Znf151</i>	32	35	Cell cycle, DNA replication, recombination, and repair, cell death
3	<i>Aplp2, Bcl2a1, Bcl2l11, Bid, Casp3, Ccnt1, Ccnt2, Cdk9, Ceacam1, Ctst, Dcc, Fem1b, Gas2, Gcnt2, Gtf2f1, Gtf2f2, His1, Hla-dqb1, Hmox2, Hnrpu, Htatsf1, Irf3, Krt8, Krt18, Mcl1, Mhc2ta, Pcaf, Ptma, Rea, Rfxank, Rfxap, Supt4h1, Supt5h, Troap, Weel</i>	32	35	Gene expression, cellular assembly and organization, hair and skin development and function
4	<i>Cd38, Clk1, Cpsf1, Ddx39, Eif1a, Eif3s4, Eif3s5, Eif3s6, Eif3s8, Eif3s10, Eif3s6ip, Eif4a2, Eif4e, Gspt1, Hmgn2, Hnrpa1, Lck, Mapk13, Mknk2, Nup214, Nxf1, Pabpc1, Paip1, Prpf4b, Rbm8a, Rps25, Ryr1, Sfrs1, Sfrs5, Srrm1, Thoc4, Thy1, Tnp3, U2af1, Ubl5</i>	32	35	Protein synthesis, RNA post-transcriptional modification, gene expression
5	<i>Atf3, Bcl3, Cbx3, Cdc2, Cdc6, Cdc25c, Cdkn2c, Cdkn2d, Chek2, Csnk2a1, Dub1, E2f6, Fgfl, Gata1, Hspa2, Junb, Lats2, Mcm3, Mcm4, Mcm6, Orc11, Paf53, Pck1, Polr1a, Polr1c, Polr1d, Pttg1, Pttg1ip, Rps10, Sei1, Smarcc1, Trim28, Ube2a, Ubtf, Zfp118</i>	32	35	Cell cycle, DNA replication, recombination, and repair, cell death
6	<i>Akap8, Arih1, Arih2, Blnk, Clqbp, Cbl, Crkl, Dck, Eif4el3, Fbl, Foxc2, Icam1, Kcnab2, Mki67ip, Msn, Ncl, Pin1, Pin4, Pip5k1a, Plec1, Ppm1a, Prkar2a, Prkca, Prkcz, Rpl10a, Rps6, Sfpq, Slc1a1, Slc2a4, Spry2, Tmpo, Top2a, Ube2l3, Vil2, Wdr12</i>	32	35	Cellular assembly and organization, carbohydrate metabolism, protein degradation
7	<i>Btg1, Clns1a, Ddx20, Dhx9, Dis3, Etv3, Fbl, Gemin5, Gemin6, Gemin7, Hrmt1l2, Ipo7, Khdrbs1, Kpnb1, Kpnb3, Mep50, Nup153, Nxf1, Ran, Ranbp2, Rangap1, Rnut1, Rpl13, Sip1, Skb1, Smndc1, Snrpb, Snrpd1, Snrpd2, Snrpd3, Snrpf, Tec, Xpo5, Xpot, Znf259</i>	18	27	RNA post-transcriptional modification, cell signaling, nucleic acid metabolism
8	<i>Arc, Coil, Ddx20, Dhx9, Fbl, Fgf2, Fkbp3, Fmr1, Gan, Gemin4, Gemin5, H2-k, Lamr1, Lgals1, Lgals3, Map1b, Mdk, Ncl, Nola1, Nola2, Nola3, Nolc1, Nop5/nop58, Nufip1, Prpf8, Rpl13a, Rps7, Sip1, Smn1, Snrpb, Snrpd2, Snrpd3, Snrpe, Snrpf, Tjft</i>	15	25	RNA post-transcriptional modification, cellular growth and proliferation, gene expression
9	<i>Apoa1, Apoe, Col11a2, Hspb1, Ipo9, Jrk, Mtl1, Myc, Mycn, Ndufa2, Pin4, Pltp, Polr2a, Polr2b, Polr2c, Polr2d, Polr2e, Polr2f, Polr2g, Polr2h, Polr2i, Polr2j, Polr2k, Polr2l, Rpl7, Rpl26, Rpl27a, Rps3, Rps7, Rps9, Rps11, Tbp, Xab2, Znf263</i>	9	19	Gene expression, lipid metabolism, cancer
10	<i>Ascl1, Axin1, Cbx3, Cbx8, Clock, Csnk1d, Csnk1e, Dbp, Ddc, Dio1, Dvl1, Dvl1l1, E2f6, H-1(3)mbt-like, Hdac3, Id1, Mbl, Ngfrap1, Per1, Pgd, Ppia, Ppp2ca, Prdx6, Ring1, Ring2, Rnf134, Rrm1, Rrm2, Rybp, Senp2, Stat5b, Thrb, Yaf2, Ywhae, Yy1</i>	9	19	Behavior, organismal development, cellular development

Statistically significant  $\alpha$ -amanitin sensitive genes from the 2-cell cultured embryos ( $\alpha$ -amanitin treated vs. untreated) were used as input for IPA analysis, and the resulting top 10 networks as well as their top 3 network function categories are shown.

### Pathway analysis

EASE analysis identified important functional categories (e.g., protein synthesis, and transcription and RNA processes) that are over-represented in the 2-cell  $\alpha$ -amanitin-sensitive transcripts. EASE provides a unique way to convert functional genomics studies from “genes to themes” (Hosack et al., 2003). To gain insight into how the individual genes in a relevant theme are interrelated or interacting with each other in a coordinated fashion to initiate and execute genome activation and concomitantly form a transcriptionally repressive state, we employed Ingenuity Pathway Analysis (IPA). IPA provides a tool to construct and extract relevant information from the microarray analysis and integrates individual genes, biological themes, and functional regulatory networks. An inherent weakness of EASE and IPA is that they are

Table 5

Top functional classes enriched in the  $\alpha$ -amanitin-sensitive genes in 2-cell cultured embryos

High-level function	Significance	# Global analysis genes
RNA post-transcriptional modification	1.75E-14–3.11E-2	58
Cell cycle	5.88E-11–4.98E-2	144
Gene expression	8.57E-9–3.14E-2	182
Protein synthesis	1.09E-8–3.47E-2	58
DNA replication, recombination, and repair	2.59E-5–3.14E-2	116
Cell death	2.55E-4–4.76E-2	208

IPA global functional analysis was used to examine the enriched functional classes of the  $\alpha$ -amanitin-sensitive gene sets in the 2-cell cultured embryos. Each of these annotated functional class listed in the left column contains multiple subcategories and the range of significance of these subcategories is listed in the middle column. The genes listed in each functional class can be found in Supplemental Table S3.

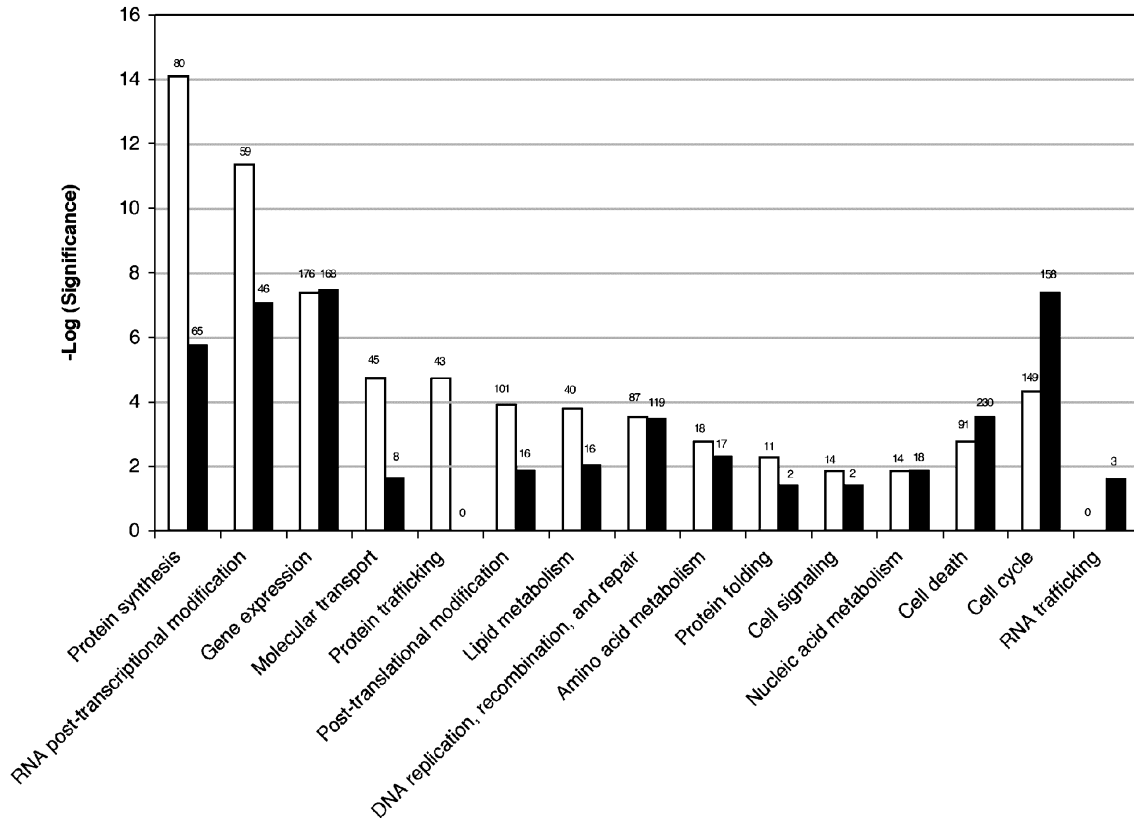


Fig. 4. Global functional category comparison of gene sets up-regulated in 2-cell embryos from in vivo or in vitro treatments. Increasing value of  $-\log$  (significance) indicates increased confidence for each category. The number of genes in each category is shown above the bars. Open bars,  $2CC>1C$  (in vivo); Solid bars,  $2CC>1CC$  (in vitro).

limited to those genes with assigned annotations or published relationships, and depend on the accuracy of these annotations.

Gene interaction networks were drawn from the lists of genes that are  $\alpha$ -amanitin-sensitive. Out of the 2607  $\alpha$ -amanitin-sensitive genes (genes whose expression was reduced by 1.7- to 276.4-fold with a FDR <5%), 791 were identified as focus genes used to generate biological networks and 899 were used for global functional analysis. Thirty-eight networks generated had a score of 3 or higher and contained 12 or more focus genes (Table 4). Notably, the top six networks identified were most significant with a score of 32 and contained the highest number of focus genes (35) allowed in each network. Because a score of 2 or higher has at least a 99% confidence of not being generated by random chance, the high confidence level associated with the large number of genes and associated networks demonstrates the extensive interrelation and interaction between the genes that are turned on at the 2-cell stage.

Listed in Table 5 are the number of genes that are in the top 6 networks and the relevant biological functions that are represented by these networks. The top functions in which these individual networks participate are consistent with the global functions generated by IPA to search for the most significant biological functions across the entire dataset of the  $\alpha$ -amanitin-sensitive genes, compared to the IPA know-

ledge base. The IPA global functional analysis (GFA) confirmed the EASE analysis that genes functioning in gene expression (20.2%) and protein synthesis (6.5%), in addition to cell cycle (16.0%) genes, are highly represented

Table 6

Top functional classes enriched in  $\alpha$ -amanitin-sensitive and up-regulated genes in in vivo developed 2-cell embryos

High level function	Significance	# Global analysis genes
RNA post-transcriptional modification	1.20E-11–3.27E-2	37
Protein synthesis	2.84E-10–2.52E-2	44
Gene expression	4.95E-7–4.72E-2	106
Cell cycle	3.56E-6–4.95E-2	72
Immune and lymphatic system development and function	2.25E-4–2.25E-4	3
Protein folding	4.40E-4–4.40E-4	9
Lipid metabolism	7.82E-4–4.95E-2	22
Cell signaling	1.12E-3–1.12E-3	9
Nucleic acid metabolism	1.12E-3–1.12E-3	9
DNA replication, recombination, and repair	1.34E-3–4.76E-2	47

IPA global functional analysis for genes that are derived from those expressed higher in the 2-cell embryos than in both 1-cell and egg in vivo, and are  $\alpha$ -amanitin-sensitive in vitro. The range of significance of the subcategory within a function is listed in the middle next to the number of global genes annotated with that function as in Table 5. The genes listed in each functional class can be found in Supplemental Table S4.

in the  $\alpha$ -amanitin-sensitive group. Because the IPA implements a similar but distinct statistical analysis tool to search for global functions in the gene set, and the reference population of annotations is also different, IPA GFA and EASE can give complementary information. IPA revealed that RNA post-transcriptional modification (6.5%), DNA replication, recombination and repair (12.9%), and cell death genes (23.1%) are also among the top six functions present in the  $\alpha$ -amanitin-sensitive gene set.

Before we implemented these analyses, we first determined the effect of embryo culture on gene expression, because such effects could adversely affect interpretation of the results. For example, genes that may be turned on in response to the culture conditions would be detected as  $\alpha$ -amanitin sensitive but would not be of biological relevance *in vivo*. Accordingly, we first compared the two sets of 2-cell, up-regulated genes that were generated from *in vivo* versus *in vitro* developed embryos to search for differences in global functional classes that these two groups of genes exhibit. This difference only served as a rough measure of the biological relevance between the two groups, considering that the *in vivo* and *in vitro* developed embryos were unlikely at precisely the same developmental stage.

Fig. 4 shows selected functional classes that are represented by these two sets of genes that are up-regulated in the 2-cell embryo when compared to the 1-cell embryo. A few classes of genes are reduced or missing in cultured embryos, and include categories for protein synthesis and processing (protein synthesis, protein trafficking, protein folding, post-translational modification), as well as other processes like RNA post-transcriptional modification. The over-representation of cell death genes may reflect a stress response to culture. Gene expression, DNA replication, and nucleic acid metabolism do not seem to be affected in terms of functional representation. We focused on exploring networks of genes whose transcripts are up-regulated in the 2-cell embryo *in vivo* and  $\alpha$ -amanitin-sensitive in order to minimize the effect of culture on our analysis.

The lists of interest that were subjected to IPA analysis were derived from those genes whose expression was higher in the 2-cell embryos than in both 1-cell embryos and eggs, besides being  $\alpha$ -amanitin-sensitive *in vitro*. We filtered the genes in the 2-cell embryo against both the 1-cell embryo and the egg to reduce the background of maternally-derived transcripts. The resulting 1759 genes were used for IPA and 465 focus genes were identified for network generation and 521 for global functional analysis. Global functional analysis once again confirmed that the highest significant functional classes in this group of genes are RNA post-transcriptional modification (7.1%), which includes genes functioning in processing of RNA such as splicing (50% of this group) and polyadenylation; protein synthesis (8.4%), gene expression (20.3%), which includes among other categories transcription (86.8% of this group); mRNA elongation, repression, expression of genes, recruitment, and DNA binding; cell cycle (13.8%), which includes genes

functioning (not exclusively) in mitotic cell cycle (66.7% of this group), G1/S transition (30.5% of this group), G2/M transition (23.6%), and DNA replication (19.4%) (Table 6). This result fits the overall biological themes of embryo development at this stage and is consistent with our previous findings (Zeng et al., 2004). A summary of the genes in the top two networks is found in Table 7.

IPA generated 25 networks with scores of 6 or higher and at least 12 focus genes in the network, including the top two networks that are most significant with a significance score of 41 and a maximum 35 focus genes (Figs. 5 and 6). Network 1 (Fig. 5) has an overall biological theme of protein synthesis, and interestingly, all focus genes centered around *c-Myc* (*Myc*). The  $\alpha$ -amanitin-sensitivity of *Myc* expression detected by the microarrays was confirmed by real-time RT-PCR. *Myc* expression increased 8.3-fold from the 1-cell to the 2-cell stage and in the presence of  $\alpha$ -amanitin this ratio was 0.38. This ratio of  $<1$  was anticipated because the zygotic *Myc* transcripts are not expressed in the  $\alpha$ -amanitin-treated embryos and maternal *Myc* transcripts continued to be degraded. It should be noted that although *Myc* expression was not called “present” in either control or treated embryos by the Affymetrix MAS filter on flags, the robustness of our experimental design revealed that these differences were statistically significant. The MAS “absent” flag likely reflects *Myc*’s low level of expression and highlights the usefulness of including lists of genes that are not necessarily called “detected” (by the MAS algorithm) for IPA as long as the differences are statistically significant.

MYC plays an important role in regulating cell cycle, cell growth, differentiation, apoptosis, transformation, genomic instability, and angiogenesis presumably by its ability to activate or repress transcription of target genes that mediate these various processes (Hipfner and Cohen, 2004). *Myc*-responsive genes have identified few overlapping target genes, except for many ribosomal genes (Boon et al., 2001). Because ribosomes are centrally involved in macromolecular synthesis and metabolism, a major proposed role for *Myc* is to regulate protein synthetic and metabolic pathways. Out of the 35 genes in Network 1 (Fig. 5), 17 are ribosomal proteins, in addition to other genes involved in ribosomal RNA biogenesis and assembly (*Ddx21* and *Nol5a*). This finding is consistent with protein synthesis and ribosome biogenesis being two major biological themes that emerge from our EASE analyses of 2-cell embryos (Zeng et al., 2004) and results described here.

The top network generated from the  $\alpha$ -amanitin-sensitive genes suggests how these functionally related genes act in a coordinated fashion, and how the up-regulation of *Myc* expression, which activates itself and hence generates an autoactivation loop, may be critical for development following genome activation. Consistent with this proposal are results from a previous study that used an anti-sense approach to ablate *Myc* function in 2-cell embryos post-ZGA and observed that virtually all of the embryos arrested at the 8-cell/morula stage (Paria et al., 1992). Moreover, our

Table 7  
Summary of genes in the top 2 IPA networks

Gene	Affymetrix	Fold Change 2C/1C	Fold Change 2CC/2CA	Networks	Description	Family	Location
<i>Atpi</i>	1448770_a_at	3.3	4.3	1	ATPase inhibitor		Cytoplasm
<i>Ddx21</i>	1448271_a_at	5.7	2.2	1	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	enzyme	Nucleus
<i>Glg1</i>	1448580_at	7.9	9.8	1	Golgi apparatus protein 1		Cytoplasm
<i>Gm2a</i>	1448241_at	7.3	12.2	1	GM2 ganglioside activator		Cytoplasm
<i>H2afz</i>	1416415_a_at	8.3	3.0	1	H2A histone family, member Z		Nucleus
<i>Hmox2</i>	1416399_a_at	4.4	2.2	1	Heme oxygenase (decycling) 2	enzyme	Cytoplasm
<i>Imp-1</i>	1418761_at	4.5	15.3	1	IGF-II mRNA-binding protein 1		
<i>Jtv1</i>	1451262_a_at	2.2	2.3	1	JTV1 gene		
<i>Lamp2</i>	1416343_a_at	72.8	11.5	1	Lysosomal-associated membrane protein 2	enzyme	Plasma Membrane
<i>Mt1a</i>	1422557_s_at	115.6	39.9	1	Metallothionein 1A (functional)		Cytoplasm
<i>Myc</i>	1424942_a_at	10.6	6.9	1, 4, 5, 7	v-myc myelocytomatosis viral oncogene homolog (avian)	transcription regulator	Nucleus
<i>Nol5a</i>	1455035_s_at	5.6	9.6	1	Nucleolar protein 5A (56kDa with KKE/D repeat)		Nucleus
<i>Pls3</i>	1423725_at	2.5	2.5	1	Plastin 3 (T isoform)		Cytoplasm
<i>Psat1</i>	1454607_s_at	198.2	152.7	1	Phosphoserine aminotransferase 1	enzyme	Cytoplasm
<i>Rpl7</i>	1415979_x_at	17.6	9.2	1, 4	Ribosomal protein L7	transcription regulator	Cytoplasm
<i>Rpl9</i>	1443843_x_at	85.5	51.9	1	Ribosomal protein L9		Cytoplasm
<i>Rpl13</i>	1460581_a_at	12.6	27.6	1	Ribosomal protein L13		Cytoplasm
<i>Rps13</i>	1415912_a_at	34.7	36.3	1	Ribosomal protein S13		Cytoplasm
<i>Rpl19</i>	1416219_at	6.9	5.7	1	Ribosomal protein L19		Cytoplasm
<i>Rps18</i>	1455572_x_at	8.2	7.7	1	Ribosomal protein S18		Cytoplasm
<i>Rpl21</i>	1429077_x_at	4.4	16.0	1	Ribosomal protein L21		Cytoplasm
<i>Rps19</i>	1449243_a_at	47.2	27.9	1	Ribosomal protein S19		Cytoplasm
<i>Rpl23</i>	1422859_a_at	37.4	6.8	1	Ribosomal protein L23		Cytoplasm
<i>Rps20</i>	1456436_x_at	19.0	13.2	1	Ribosomal protein S20		Cytoplasm
<i>Rpl27</i>	1448217_a_at	6.9	4.0	1	Ribosomal protein L27		Cytoplasm
<i>Rps23</i>	1460175_at	18.7	9.6	1	Ribosomal protein S23		Cytoplasm
<i>Rpl30</i>	1418273_a_at	6.7	6.3	1	Ribosomal protein L30		Cytoplasm
<i>Rps27</i>	1415716_a_at	20.5	16.1	1	Ribosomal protein S27 (metallopanstimulin 1)		Cytoplasm
<i>Rpl35</i>	1436840_x_at	6.9	5.0	1	Ribosomal protein L35		Cytoplasm
<i>Rps4x</i>	1416276_a_at	7.1	6.0	1	Ribosomal protein S4, X-linked		Cytoplasm
<i>Rpl41</i>	1455578_x_at	31.4	5.0	1	Ribosomal protein L41		Cytoplasm
<i>Sasdh</i>	1416662_at	4.4	15.8	1	Sarcosine dehydrogenase	enzyme	Cytoplasm
<i>Shmt1</i>	1425179_at	72.2	33.4	1	Serine hydroxymethyltransferase 1 (soluble)	enzyme	Cytoplasm
<i>Tde1</i>	1417815_a_at	5.1	4.3	1	Tumor differentially expressed 1		Plasma Membrane
<i>Znf151</i>	1416224_at	5.7	9.5	1	Zinc finger protein 151 (pHZ-67)		Nucleus
<i>Apex1</i>	1416135_at	2.6	2.3	2	APEX nuclease (multifunctional DNA repair enzyme) 1	enzyme	Nucleus
<i>Asid4a</i>	1436191_at	2.7	5.5	25, 2	AT-rich interactive domain 4A (RBP1-like)	transcription regulator	Nucleus
<i>Bcos</i>	1428773_s_at	4.1	6.0	2	BCL6 co-repressor	transcription regulator	Nucleus
<i>Ccne1</i>	1416492_at	15.9	10.8	19, 2	Cyclin E1		Nucleus
<i>Ccne2</i>	1422535_at	3.4	3.5	2	Cyclin E2		Nucleus
<i>Cdc2</i>	1448314_at	6.1	5.7	2, 13	Cell division cycle 2, G1 to S and G2 to M	kinase	Nucleus
<i>Cdc6</i>	1417019_a_at	3.5	5.5	2	CDC6 cell division cycle 6 homolog ( <i>S. cerevisiae</i> )		Nucleus
<i>Cdc25c</i>	1422252_a_at	61.8	17.7	2	Cell division cycle 25C	phosphatase	Nucleus
<i>Cdk5s1</i>	1433451_at	6.2	16.1	2	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)		Nucleus
<i>Cdkn1a</i>	1421679_a_at	6.8	6.8	2	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)		Nucleus
<i>Chek2</i>	1422747_at	6.3	4.6	2	CHK2 checkpoint homolog ( <i>S. pombe</i> )	kinase	Nucleus
<i>Cul3</i>	1422794_at	9.4	4.6	2	Cullin 3		Nucleus

Table 7 (continued)

Gene	Affymetrix	Fold Change 2C/1C	Fold Change 2CC/2CA	Networks	Description	Family	Location
<i>Fkbp3</i>	1416859_at	73.8	29.1	2	FK506 binding protein 3, 25 kDa	enzyme	Nucleus
<i>Gadd45a</i>	1449519_at	5.3	24.5	2, 6	Growth arrest and DNA-damage-inducible, alpha		Nucleus
<i>Gadd45b</i>	1450971_at	10.9	14.1	2	Growth arrest and DNA-damage-inducible, beta		Cytoplasm
<i>Hdac1</i>	1448246_at	16.4	8.0	2	Histone deacetylase 1	transcription regulator	Nucleus
<i>Klf4</i>	1417394_at	8.6	6.2	2	Kruppel-like factor 4 (gut)	transcription regulator	Nucleus
<i>Madh7</i>	1423389_at	6.6	6.8	2	SMAD, mothers against DPP homolog 7 ( <i>Drosophila</i> )		Nucleus
<i>Mizf</i>	1440255_at	3.6	2.3	2	MBD2 (methyl-CpG-binding protein)-interacting zinc finger protein		Nucleus
<i>Mta2</i>	1423165_a_at	8.0	15.2	25, 2	Metastasis-associated gene family, member 2	transcription regulator	Nucleus
<i>Nme2</i>	1448808_a_at	181.6	50.5	2	Non-metastatic cells 2, protein (NM23B) expressed in	kinase	Nucleus
<i>Pim1</i>	1435458_at	3.2	2.6	2	pim-1 oncogene	kinase	Cytoplasm
<i>Ppia</i>	1417451_a_at	4.0	2.0	2	Peptidylprolyl isomerase A (cyclophilin A)	enzyme	Cytoplasm
<i>Pspf4b</i>	1455696_a_at	15.1	6.4	2	PRP4 pre-mRNA processing factor 4 homolog B (yeast)	kinase	Nucleus
<i>Pttg1</i>	1419620_at	9.2	12.8	2	Pituitary tumor-transforming 1	transcription regulator	Nucleus
<i>Rnf7</i>	1426414_a_at	8.8	10.4	2	Ring finger protein 7		Nucleus
<i>Rps10</i>	1434854_a_at	4.8	4.1	2	Ribosomal protein S10		Cytoplasm
<i>Set</i>	1421819_a_at	28.9	4.5	2	SET translocation (myeloid leukemia-associated)		Nucleus
<i>Sfss1</i>	1434972_x_at	20.2	4.0	2	Splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)		Nucleus
<i>Sfss5</i>	1423130_a_at	3.1	2.9	2	Splicing factor, arginine/serine-rich 5		Nucleus
<i>Tnpo3</i>	1429369_at	16.2	18.5	2	Transportin 3		
<i>Tob1</i>	1423176_at	21.0	10.5	2	Transducer of ERBB2, 1	transcription regulator	Cytoplasm
<i>Top2a</i>	1454694_a_at	3.1	5.0	2	Topoisomerase (DNA) II alpha 170 kDa	enzyme	Nucleus
<i>Ube2a</i>	1448772_at	7.3	4.5	2	Ubiquitin-conjugating enzyme E2A (RAD6 homolog)	enzyme	Cytoplasm
<i>Yy1</i>	1422569_at	25.4	18.7	2	YY1 transcription factor	transcription regulator	Nucleus

finding that *Myc* is identified in six significant networks (with a score of 9 and higher) that represent many different biological themes, as well as the ability to activate or repress either directly or indirectly many core genes in the other networks (e.g., the cell cycle regulatory genes cyclin E, *Cdc2* in Network 2) demonstrates the extent that *Myc* can regulate multiple subsets of genes to elicit specific genetic programs. Thus, once the genome is activated, presumably by maternally-derived components, *Myc* may be a seminal component involved in expression of numerous genes that are critical for continued development.

Superimposed on genome activation is the development of a chromatin-based transcriptional repressive state in the 2-cell embryo (see Introduction) that is relieved by inducing histone hyperacetylation. This finding implicates histone deacetylases (HDACs) as major players responsible for the repressive state, but which member(s) of the *Hdac* family

are responsible has remained elusive, because the HDAC inhibitors used were not isoform-specific (Aoki et al., 1997; Davis et al., 1996; Henery et al., 1995; Wiekowski et al., 1993). Our results suggest that expression of *Hdac1* may be central to the development of the transcriptionally repressive state.

HDAC1 has the most number of interactions (11) with other focus genes in Network 2, which has an overall functional theme for cell cycle and DNA replication (Fig. 6). Expression profiles of all the HDACs present on the Affymetrix MOE430 GeneChips (including *Hdac1*, 2, 3, 5, 6, 7a, 8, 10, and 11) reveals that only *Hdac1* is  $\alpha$ -amanitin-sensitive, a finding confirmed by real-time RT-PCR. *Hdac1* abundance increases 1.6-fold between the 1-cell and 2-cell stages, but that this ratio is 0.1 when  $\alpha$ -amanitin is present; as with *Myc*, the ratio of less than 1 was anticipated and likely reflects degradation of maternal *Hdac1* transcripts.

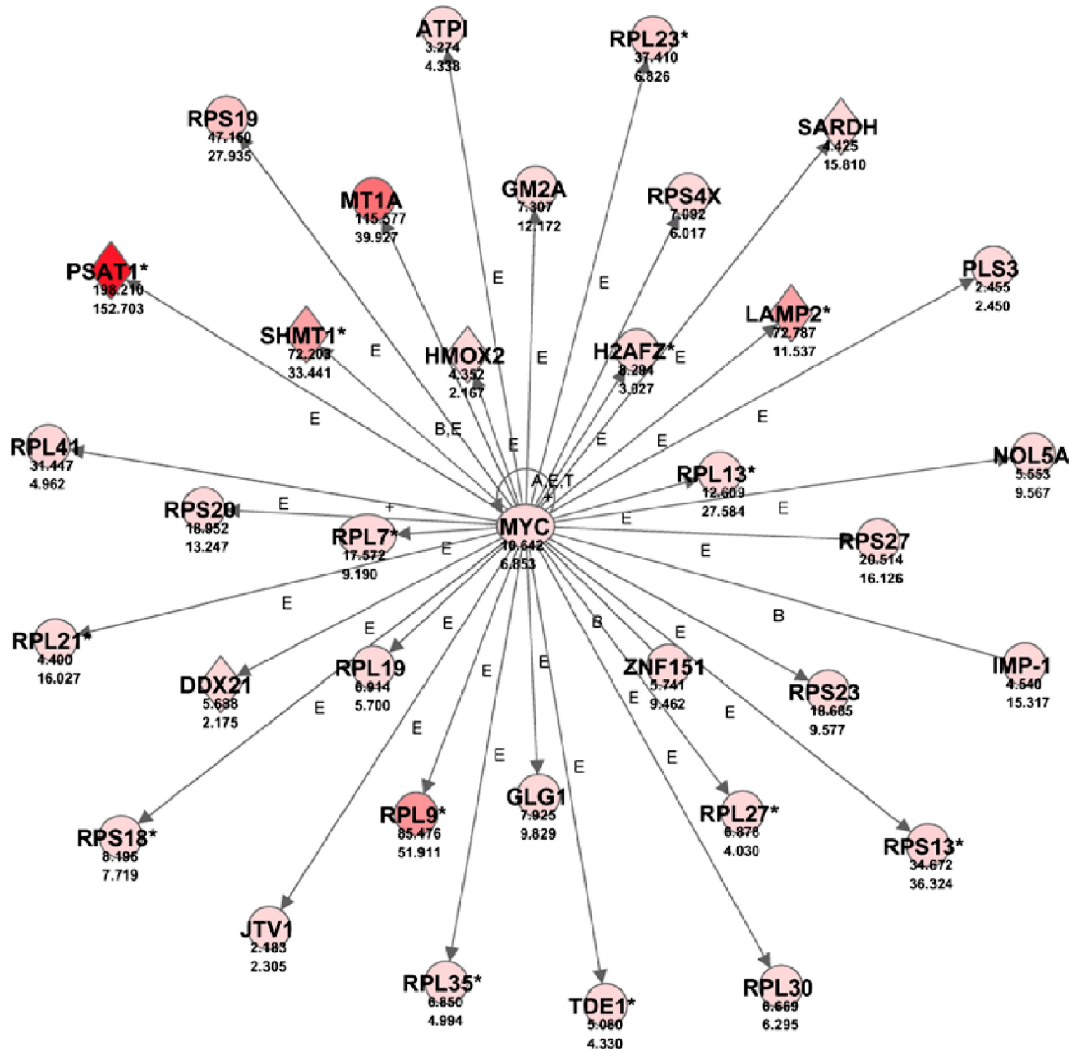


Fig. 5. Top interaction Network 1. Network 1 is from IPA analysis of gene sets up-regulated in 2-cell embryos compared to 1-cell embryos and eggs and are also  $\alpha$ -amanitin-sensitive. The network is displayed graphically as nodes (gene or gene product) and edges (the biological relationships between nodes, including functional or physical interactions, e.g., E, expression; B, binding). The numbers under a node are the fold-change of 2C over 1C (up-regulation, top number) and 2CC over 2CA ( $\alpha$ -amanitin-sensitive, bottom number). See Table 7 for a complete description of the genes. The shape of the objects (e.g., circle, diamond) represents whether the protein is a structural protein, transcription factor, etc. and the family classes represented by these shapes are listed in Table 7. The greater the color intensity, the greater the up-regulation in the 2-cell relative to the 1-cell embryo in vivo.

Furthermore, additional candidate genes are also implicated by this network and include, among the 11 genes that show physical interaction with HDAC1: MTA2, which is a component of the nucleosome remodeling deacetylase complex NuRD (which also includes HDAC1) that is involved in chromatin assembly/disassembly (Ng and Bird, 2000); ARID4A, a nuclear protein that can serve as a bridging molecule to recruit HDACs and provides a second HDAC-independent repression function (Wilsker et al., 2002); BCOR (BCL6 co-repressor) that selectively interacts with transcription repressor BCL6 and can bind to specific class I and II HDACs (Bertos et al., 2001); MIZF interacts with MBD2, which is a component of MeCP1 (MBD2)–HDAC complex and plays a role in DNA methylation and transcription repression (Sekimata and Homma, 2004; Sekimata et al., 2001); and YY1 (Yin Yang 1), which

activates or represses transcription by binding with many other factors (Thomas and Seto, 1999), and interestingly, translocates from the cytoplasm of 1-cell embryos to the nucleus in 2-cell embryos (Donohoe et al., 1999), that is, when the transcriptionally repressive state develops. The  $\alpha$ -amanitin-sensitivity of *Mta2* and *Yy1* was confirmed by real-time RT-PCR (data not shown).

The network data, functions, and interactions to build these networks were generated from different experimental systems, such as cell-free systems, in vitro culture of normal or cancer cells, and/or in other animal models, and hence the applicability of these results in the preimplantation embryo awaits validation. Nevertheless, the results presented here that employ microarray analysis of gene expression, coupled with EASE and IPA, highlight the power of this hypothesis-generating approach to identify candidate genes that

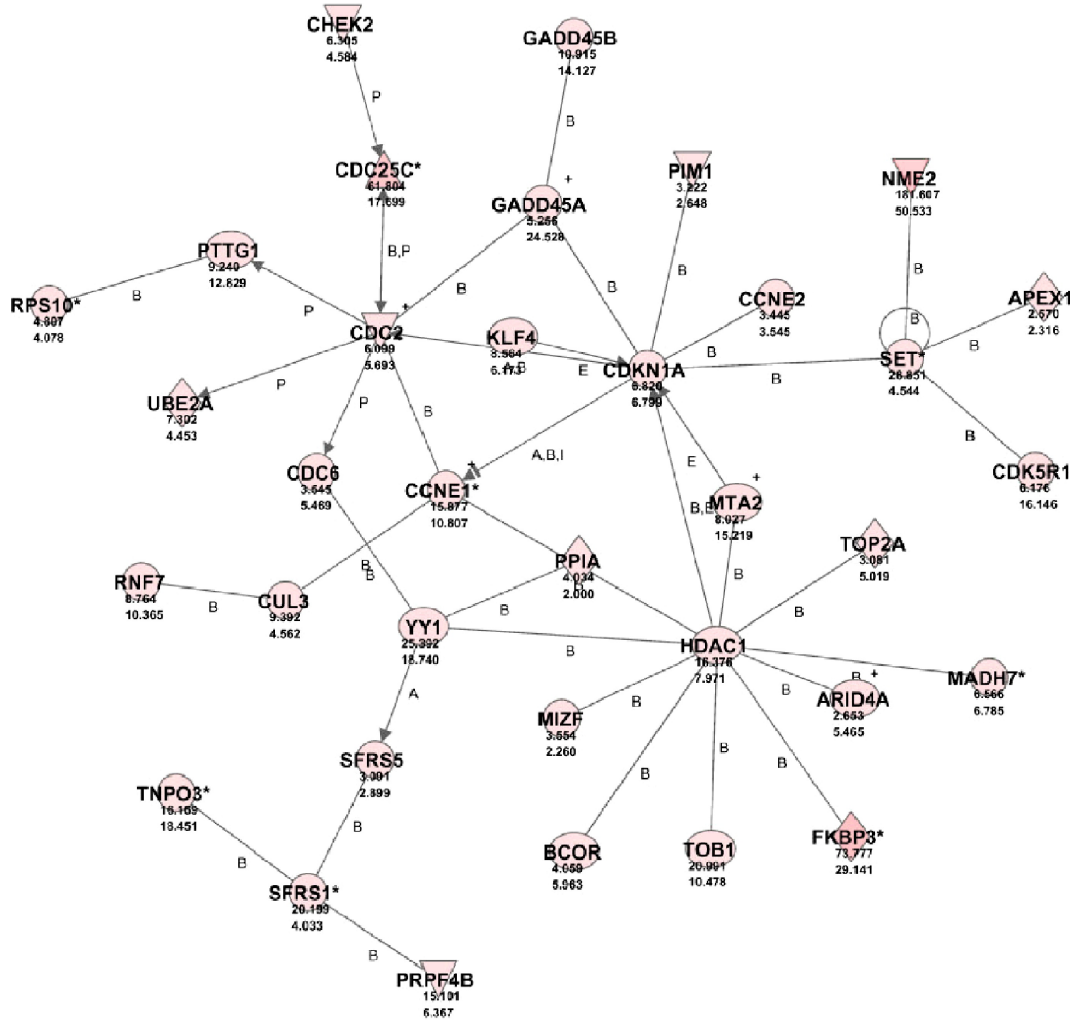


Fig. 6. Top interaction Network 2. Network 2 is from IPA analysis of gene sets up-regulated in 2-cell embryos compared to 1-cell embryos and eggs and is also  $\alpha$ -amanitin-sensitive. See legend to Fig. 5 for details.

will be the subject of a hypothesis-driven approach. We are currently pursuing the role of *Myc* and *Hdac1* as critical players in genome activation and repression, respectively, in the preimplantation mouse embryo.

*Cross-platform analysis*

There is a growing concern about the reproducibility of data sets obtained from microarray experiments, and the ability to merge existing data sets, especially when the experiments used different platforms (Marshall, 2004). To determine the concordance in  $\alpha$ -amanitin-sensitive genes that we detected to that described in another study that used long oligos based on preimplantation and post-implantation, and ovarian cDNA libraries (Hamatani et al., 2004), we first identified the common set of genes expressed in 2-cell embryos and then determined the fraction of  $\alpha$ -amanitin-sensitive genes common to both sets (Table 8). Note that although 2990 Affymetrix MOE430 probe sets are up-regulated by at least 1.4-fold in the 2-cell embryo, only 15% are present in the up-regulated NIA set. This complicates a

cross-platform comparison because different genes are being assayed with different platforms. Despite the differences in both experimental design and the criteria employed to identify  $\alpha$ -amanitin-sensitive genes, the concordance was quite high in the set of commonly up-regulated genes. For

Table 8  
Cross-platform analysis of  $\alpha$ -amanitin-sensitive genes in 2-cell embryos

	MOE430 2CC>1CC (>1.4 $\times$ )		MOE430 2CC>1CC (>5 $\times$ )	
	NIA+	NIA-	NIA+	NIA-
MOE+	288 (69.6%)	12 (2.9%)	179 (84.0%)	9 (4.2%)
MOE-	110 (26.6%)	4 (1.0%)	24 (11.3%)	1 (0.5%)

MOE430 2CC>1CC (>1.4 $\times$ ) refers to genes that are up-regulated by at least a 1.4-fold between 1-cell and 2-cell stages in the MOE set that are common in the MOE430 and combined NIA set. MOE430 2CC>1CC (>5 $\times$ ) refers to genes that are up-regulated by at least a 5-fold in the MOE set between the 1-cell and 2-cell stages that are common in the MOE and combined NIA set. MOE+ and NIA+ are genes that are  $\alpha$ -amanitin-sensitive, whereas MOE- and NIA- are genes that are  $\alpha$ -amanitin-insensitive. The number of genes in each category is shown and the numbers in parentheses correspond to the fraction of genes in this category when compared to the total number in all four categories.



example, using genes that displayed only a 1.4-fold increase between the 1-cell and 2-cell stages (Table 2), ~70% of the commonly detected genes in 2-cell embryos are also  $\alpha$ -amanitin-sensitive. When at least a 5-fold increase in expression between the 1-cell and 2-cell stages (Table 2) was employed, the overlap in  $\alpha$ -amanitin-sensitive genes was 84%. The common set of  $\alpha$ -amanitin-sensitive genes is found in Table S6. The consistency of these results between the two studies provides reassurance that using microarrays to analyze gene expression in preimplantation mouse embryos has been and will be a valid and powerful approach to study gene expression during preimplantation development.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ydbio.2005.03.038](https://doi.org/10.1016/j.ydbio.2005.03.038).

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