Molecular signature of anastasis for reversal of apoptosis
[version 2; referees: 3 approved]

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Abstract
Anastasis (Greek for "rising to life") is a cell recovery phenomenon that rescues dying cells from the brink of cell death. We recently discovered anastasis to occur after the execution-stage of apoptosis in vitro and in vivo. Promoting anastasis could in principle preserve injured cells that are difficult to replace, such as cardiomyocytes and neurons. Conversely, arresting anastasis in dying cancer cells after cancer therapies could improve treatment efficacy. To develop new therapies that promote or inhibit anastasis, it is essential to identify the key regulators and mediators of anastasis – the therapeutic targets. Therefore, we performed time-course microarray analysis to explore the molecular mechanisms of anastasis during reversal of ethanol-induced apoptosis in mouse primary liver cells. We found striking changes in transcription of genes involved in multiple pathways, including early activation of pro-cell survival, anti-oxidation, cell cycle arrest, histone modification, DNA-damage and stress-inducible responses, and at delayed times, angiogenesis and cell migration. Validation with RT-PCR confirmed similar changes in the human liver cancer cell line, HepG2, during anastasis. Here, we present the time-course whole-genome gene expression dataset revealing gene expression profiles during the reversal of apoptosis. This dataset provides important insights into the physiological, pathological, and therapeutic implications of anastasis.
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Amendments from Version 1

In the revised manuscript, we have added new data that support our conclusions. Specifically, our RT-PCR reveals that a human liver cancer cell line displays similar gene expression profile during anastasis as observed in mouse primary liver cells. Additional microarray statistical analysis is included as supplementary data. We have also discussed the potential molecular mechanisms, physiological and pathological consequences, and therapeutic potentials of anastasis.

See referee reports

Introduction

Apoptosis (Greek for “falling to death”) is essential for normal development and homeostasis of multicellular organisms by eliminating unwanted, injured, or dangerous cells\(^3\)–\(^9\). This cell suicide process was generally assumed to be irreversible because it involves rapid and massive cell destruction\(^9\). During apoptosis, intrinsic and extrinsic pro-apoptotic signals can converge at mitochondria, leading to mitochondrial outer membrane permeabilization (MOMP), which releases cell execution factors, such as cytochrome c to trigger activation of apoptotic proteases including caspase-3 and -7\(^1\)\(^1\)\(^3\)\(^4\), small mitochondria-derived activator of caspases (Smac)/direct IAP binding protein with low pi (DIABLO) to eliminate inhibitor of apoptosis protein (IAP) which suppresses caspase activation\(^1\)\(^3\)\(^4\), and apoptosis-inducing factor (AIF) and endonuclease G to destroy DNA\(^1\)\(^5\)\(^6\)\(^7\). Activated caspases commit cells to destruction by cleaving hundreds of functional and structural cellular substrates\(^8\)–\(^9\). Crosstalk between signalling pathways amplifies the caspase cascade to mediate cell demolition via nucleases (DNA fragmentation factor [DFF]/caspase-activated DNase [CAD]) to further destroy the genome\(^1\)\(^6\)–\(^2\)\(^1\), and alter lipid modifying enzymes to cause membrane blebbing and apoptotic body formation\(^2\)\(^2\)–\(^2\)\(^3\). Therefore, cell death is considered to occur after caspase activation within a few minutes\(^2\)\(^4\)–\(^2\)\(^6\).

However, we and other groups have demonstrated reversal of early stage apoptosis, such as externalization of phosphatidylserine (PS) in cultured primary cells and cancer cell lines\(^2\)\(^7\)–\(^2\)\(^9\). We have further demonstrated that dying cells can reverse apoptosis even after reaching the generally assumed “point of no return”\(^2\)\(^8\)–\(^2\)\(^9\), such as MOMP-mediated cytochrome c release, caspase-3 activation, DNA damage, nuclear fragmentation, and apoptotic body formation\(^2\)\(^1\)\(^0\)–\(^2\)\(^1\). Our observation of apoptosis reversal at late stages is further supported by an independent study, which shows recovery of cells after MOMP\(^2\)\(^1\). To detect reversal of apoptosis in live animals, we have further developed a new in vivo caspase biosensor, designated “CaspaseTracker”\(^2\)\(^1\), to identify and track somatic, germ and stem cells that recover after transient cell death inductions, and also potentially during normal development and homeostasis in Drosophila melanogaster after caspase activation\(^3\)\(^3\)–\(^3\)\(^4\), the hallmark of apoptosis\(^3\)\(^5\). We proposed the term “anastasis”\(^3\)\(^6\), which means “rising to life” in Greek, to describe this recovery from the brink of cell death. Anastasis appears to be an intrinsic cell survival phenomenon, as removal of cell death stimuli is sufficient to allow dying cells to recover\(^2\)\(^8\)–\(^2\)\(^9\)\(^3\)\(^1\)–\(^3\)\(^2\).

The physiological, pathological and therapeutic importance of anastasis is not yet known. We proposed that anastasis could be an unexpected tactic that cancer cells use to escape cancer therapy\(^2\)\(^9\)–\(^3\)\(^1\). Many tumours undergo dramatic initial responses to cell death-inducing radiation or chemotherapy\(^3\)\(^6\)–\(^3\)\(^9\); however, these cells relapse, and metastasis often occurs in most types of cancer\(^3\)\(^6\)–\(^3\)\(^9\). Therefore, the ability of cells to recover from transient induction of cell death may allow tumour cells to escape treatment, and survive and proliferate, resulting in relapse\(^3\)\(^6\)–\(^3\)\(^1\). Furthermore, cells may acquire new oncogenic mutations and transformation phenotypes during anastasis\(^3\)\(^6\)–\(^3\)\(^1\), such as DNA damage caused by apoptotic nucleases. Therefore, anastasis could be one of the mechanisms underlying the observation that repeated tissue injury increases the risk of cancer in a variety of tissues\(^4\)\(^0\), such as liver damage due to alcoholism\(^4\)\(^1\), chronic thermal injury in the oesophagus induced by the consumption of very hot beverages\(^4\)\(^2\)–\(^4\)\(^4\), evolution of drug resistance in recurrent cancers\(^3\)\(^6\)–\(^3\)\(^9\)\(^4\)\(^5\), and development of a second cancer during subsequent therapy\(^4\)\(^6\)–\(^4\)\(^9\). Anastasis can also occur in primary cardiac cells and neuronal cell lines\(^3\)\(^6\)–\(^3\)\(^1\) and potentially in cardiomyocytes in vivo following transient ischemia\(^4\)\(^0\). These findings suggest anastasis as an unexpected cellular protective mechanism.

Therefore, uncovering the mechanisms of anastasis may provide new insights into the regulation of cell death and survival, and harnessing this mechanism via suppression or promotion of anastasis would aid treatment of intractable diseases including cancer, heart failure and neurodegeneration.

Our previous study demonstrated reversibility of ethanol-induced apoptosis at late stages in mouse primary liver cells, and revealed that new transcription is important to reverse apoptosis\(^3\)\(^9\)–\(^4\)\(^1\). During recovery, we found up-regulation of genes involved in pro-survival pathways and DNA damage responses during anastasis (Bag3, McI1, Dnajb1, Dnajb9, Hsp90aa1, Hspa1b, and Hspb1, Mdm2)\(^4\)\(^2\). Interestingly, inhibiting some of those genes by corresponding specific chemical inhibitors significantly suppresses anastasis\(^3\)\(^9\). However, the molecular mechanism of anastasis remains to be elucidated. To study the cellular processes of anastasis, we performed time-course RNA microarray analysis to determine the gene expression profiles of the cultured mouse primary liver cells undergoing anastasis following transient exposure to ethanol that triggers apoptosis, and identified unique gene expression patterns during reversal of apoptosis. We also performed reverse transcription polymerase chain reaction (RT-PCR) to validate the gene expression patterns in the human liver cancer cell line, HepG2, during anastasis. Here, we present our time-course microarray data, which reveals the molecular signature of anastasis.

Methods

Microarray

Mouse primary liver cells were isolated from BALB/c mice using collagenase B and cultured as described\(^4\)\(^3\)–\(^4\)\(^5\). The cells were cultured in in DMEM/F-12 (DMEM:nutrient mixture F-12) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Carlsbad, CA, USA) at 37 °C under an atmosphere of 5% CO\(_2\)/95% air. To induce apoptosis, cells were exposed to 4.5% ethanol for 5 hours (R0) in...
the culture medium (vol/vol). To allow recovery, dying cells were washed and further incubated in the fresh culture medium for 3 hours (R3), 6 hours (R6), 24 hours (R24), and 48 hours (R48). The untreated cells served as control (Ctrl). Three biological replicates were performed at each time point. Total RNA in the corresponding cell conditions was harvested using TRIzol Reagent (Life Technologies). The RNA was purified using the RNeasy Mini Kit (Qiagen, Cologne, Germany). Reverse transcription was performed using SABiosciences C-03 RT² First Strand Kit to construct cDNA (SABiosciences-Qiagen, Frederick, MD, USA). The cDNA samples were analysed using the Illumina MouseWG-6 v2.0 Expression BeadChip (Illumina, San Diego, CA, USA).

Gene expression data analysis

The Partek Genomics Suite 6.6 (Partek, St. Louis, MO, USA) was used for principal component analysis (PCA)\cite{PCR1, PCR2}. The Spotfire DecisionSite 9.1.2 (TIBCO, Palo Alto, CA, USA) platform was used to evaluate the fold change of gene expression levels between time points when compared with a common starting point, which is the control (Ctrl)\cite{RTA}. Signal values were converted into log, space and quality control tests were performed to ensure data integrity by comparing the signals of the three biological replicates at each time point. The fold change was based on averaged values of the three replicates at each time point; two-sample Student’s t-test was used to determine statistical significance as p-values of less than 0.05, using the Partek Genomics Suite v6.5 (Partek Inc., St. Louis, MO, USA).

For the time-course gene expression analysis using Spotfire, all time points were compared with the time point Ctrl, which represents untreated cells. Spotfire was used to show the genes that displayed specific changes in gene expression after removal of cell death inducer for 3 hours (R3) and 6 hours (R6). Genes with specific and significant change (Log2 > 1 or < -1) in expression at the corresponding timepoint are highlighted. Interaction network analysis of the up-regulated genes during anastasis was performed using the GeneMANIA database (http://genemania.org/v3.56).

Confocal microscopy

Cells were incubated with 50 nM Mitotracker Red CMXRos and 250 ng/ml Hoechst 33342 (Invitrogen) for 20 minutes in culture medium to stain mitochondria and nuclei, respectively. The stained cells were washed and incubated with culture medium for 10 minutes, and then were fixed with 3.7% (wt/vol) paraformaldehyde in phosphate-buffer saline (PBS) solution for 20 minutes at room temperature in dark. The fixed cells were mounted on glass slide using ProLong Diamond Antifade Mountant (Invitrogen). Cell images were captured with the Zeiss LSM 780 confocal inverted microscope using a 40x, numerical aperture (NA) 1.4 plan-Apochromat objective (Carl Zeiss, Jena, Germany), and were analyzed using Zen 2013 or AxioVision 4.2 software (Carl Zeiss).

Reverse transcription polymerase chain reaction (RT-PCR)

Human liver cancer cell line HepG2 (ATCC HB-8065) was cultured in DMEM/F-12, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies) at 37°C under an atmosphere of 5% CO2/95% air. Apoptosis was induced by incubation of the cells with 4.5% ethanol in cell culture medium for 5 hours (R0). Then, the apoptotic dying cells were washed and then incubated in the fresh culture medium for 1 hour (R1), 2 hours (R2), 3 hours (R3), 4 hours (R4), 6 hours (R6), 9 hours (R9), 12 hours (R12), and 24 hours (R24). The untreated cells served as control (Ctrl). Total RNA in the corresponding cell conditions was harvested using QIAzol lysis reagent (Qiagen). The total RNA was purified using the RNeasy Mini Kit (Qiagen). Reverse transcription was performed using the SuperScript IV reverse transcriptase system (Thermo Fisher Scientific, Waltham, MA, USA). Primer sets for detecting targeted genes were designed using the Universal ProbeLibrary (Roche Applied Science, Madison, WI). Primer set for MMP10 was previously designed\cite{RTB}. Polymerase Chain Reaction (PCR) was performed using Taq DNA Polymerase and PCR protocol (New England Biolabs, Ipswich, MA, USA), with initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 3 seconds. Electrophoresis of PCR products was performed using 4% agarose gel.

Results and discussion

We have demonstrated that mouse primary liver cells can reverse the apoptotic process at the execution stage\cite{RTD}, despite reaching important checkpoints commonly believed to be the “point of no return”\cite{RTE, RTF}, including caspase-3 activation, DNA damage, and cell shrinkage. To pursue the mechanisms of anastasis, we performed time-course high-throughput microarray to evaluate gene expression profiles during reversal of ethanol-induced apoptosis in mouse primary liver cells. RNA samples were collected from the untreated primary liver cells (Ctrl), the cells treated with 4.5% ethanol for 5 hours when cells exhibited hallmarks of apoptosis (R0), and the treated cells that were then washed and cultured in fresh medium for 3 (R3), 6 (R6), 24 (R24) and 48 (R48) hours. Apoptosis was confirmed in the ethanol-treated cells (R0), which displayed hallmarks of apoptosis, including plasma membrane blebbing, cell shrinkage, cleavage of caspase-3 and its substrates, such as PARP and ICAD (Figure 1A and B, images reprinted with permission\cite{RTG}). The features of apoptosis vanished after removal of the cell death inducer (R24), indicating recovery of the cells (Figure 1A and B). Three biological replicates were performed at each time point. The principal component analysis indicated that all three biological replicates of each time point exhibited a very high correlation, as indicated by clustering, for the dataset of all 18 samples (Figure 2A; Supplementary Figure 1; see Data availability). The unsupervised hierarchical clustering confirms the similarity between all the replicates at each time point (Figure 2B; see Data availability; Supplementary Figure 2).

Genes that display significant changes in expression during anastasis at the earliest time point of 3 hours, following the removal of the cell death inducer, may represent critical first responders of anastasis (Figure 3A, Table 1), including transcription factors of the activator protein-1 (AP-1) family (Afp3, Fos, Fosb, Jun, Junb), transforming growth factor-β (TGF-β) signal pathway and its related regulators (Inhba, Snai1, Tgfl1, Sxox4, Sxox9, Klf4, Klf6, Klf9), pro-survival Bcl-2 family member (Bag3),
inhibitor of p53 (Mdm2), anti-oxidation (Hmox1), anti-proliferation (Btg1), DNA damage (Ddit3, Ddit4), vesicular trafficking (Vps37b) and stress-inducible (Dnajb1, Dnajb9, Herpud1, Hspb1, Hspa1b) responses. Starting at 6 hours of anastasis, other groups of gene pathways display the peak of transcription, such as cell cycle arrest (Cdkn1a, Trp53inp1), autophagy (Atg12, Sqstm1), and cell migration (Mmp9, Mmp10 and Mmp13) (Figure 3B, Table 1 and Table 2). Expression of potent angiogenic factors, such as Vegfa and Angptl4, are up-regulated at 3 and 6 hours of anastasis, respectively (Table 1 and Table 2). Histones display up- (Hist1h2ae, H2afj) and down- (Hist1h2ak, Hist1h2ag, Hist1h2ap, Hist1h2af, Hist2h2ac, Hist1h2ah) regulations during the first 6 hours of anastasis (Table 2 and Table 3). Changes in expression of most of these genes peak at the 3–6-hour time points after removal of the apoptotic stimulus and then return to baseline (Figure 3A and B; Supplementary Figure 2). Interestingly, certain genes such as splicing of pre-mRNA (Rnu6), and growth arrest and DNA repair (Gadd45g) stay up-regulated during both apoptosis and anastasis (Figure 3C, Table 4).

We further observed the similar changes in gene expressions during anastasis in cultured human liver cancer HepG2 cells (Figure 4; see Data availability). The untreated HepG2 cells displayed tubular and filamentous mitochondria in the cells that spread on the substrate (Figure 4Ai, 4B). After exposure to 4.5% ethanol for 5 hours, the treated cells displayed morphological hallmarks of apoptosis, such as mitochondrial fragmentation, nuclear condensation, plasma membrane blebbing, and cell shrinkage (Figure 4Ai, 4B). After washed and incubated with fresh culture medium, the treated cells regained normal morphology (Figure 4Aii, 4B). Interestingly, the HepG2 cells that underwent anastasis displayed the increase in micronuclei formation (Figure 4B), which is the biomarker of DNA damage36, as we previously observed in mouse primary liver cells, mouse embryonic fibroblast NIH 3T3 cells, human cervical cancer HeLa cells, and human small cell lung carcinoma H446 cells55–57. By using reverse transcription polymerase chain reaction (RT-PCR), we verified our microarray data on HepG2 cells during reversal of ethanol-induced apoptosis, and found similar gene expression patterns during anastasis, including changes in mRNA levels of ANGPTL4, ATF3, ATG12, CDKN1A, FOS, HSPA1B, JUN, MDM2, MMP10 and SOX9 (Figure 4C; Supplementary Figure 3). This suggests that the mechanism of anastasis is conserved between primary and cancer cells.

The change in transcriptional profiles during anastasis provides us mechanistic insights into how dying cells could reverse apoptosis (Figure 5). In early anastasis, our data reveals that the regulators of the TGF-β signalling pathway, which control various fundamental cellular and pathological process, including proliferation, cell survival, apoptosis, cell migration, and transformation, are upregulated. The activation of the TGF-β pathway is further supported by the upregulation of AP-1 (Jun-Fos), as observed here during early anastasis. The up-regulation of the TGF-β pathway can also promote the expression of murine double minute 2 (Mdm2)53–55, an inhibitor of p53 that is also up-regulated during early anastasis56. As p53 plays a critical role in regulating apoptosis and DNA repair54,55, the expression of Mdm2 could not only promote cell survival by inhibiting p53-mediated cell death, but also cause mutations as we have observed in the cells after anastasis56. Expression of Mdm2 can also activate XIAP57, which inhibits caspases 3, 7 and 958–60, and therefore, could promote anastasis by suppressing the caspase-mediated cell destruction process. Up-regulation of anti-apoptotic BCL2 protein (Bag3) and heat shock proteins (Hsps) during anastasis can also neutralize pro-apoptotic proteins to promote cell recovery61,62. Expression of Hmox1, which encodes heme oxygenase61,
Figure 2. Technical validation of microarray data. The three biological replicate samples of microarray data were shown to cluster together by using (A) principal component analysis (PCA) and (B) unsupervised hierarchical clustering of the RNA microarray data of eighteen samples.
Figure 3. Change of gene expression profiles during reversal of apoptosis in mouse primary liver cells. Log$_2$-fold change of gene expression comparison between untreated cells (Ctrl), ethanol-induced apoptotic cells (R0), and induced cells that were then washed and further cultured in fresh medium for 3 (R3), 6 (R6), 24 (R24), and 48 (R48) hours. Genes that displayed specific (A) up-regulation at R3, (B) up- or down-regulation at R6, and (C) up-regulation anytime during the period from R0 to R6 with absolute log$_2$ fold change >1 are highlighted. The log$_2$ signal values from three biological replicates were averaged (geometric mean) for each time point.
Table 1. List of top 67 up-regulated genes at 3rd hour (R3) of anastasis, with log₂ fold change >1, compared with Ctrl (untreated cells).

| Sort Order | Gene Symbol | Definition | Accession | Log₂ fold change R3 vs. Ctrl |
|------------|-------------|------------|-----------|-----------------------------|
| 1          | Atf3        | activating transcription factor 3 | NM_007498.2 | 4.08867 |
| 2          | Hspa1b      | heat shock protein 1B | NM_010478.2 | 3.88264 |
| 3          | Fosb        | FBJ osteosarcoma oncogene B | NM_008036.2 | 3.40725 |
| 4          | Fos         | FBJ osteosarcoma oncogene | NM_010234.2 | 3.03649 |
| 5          | Egr2        | no definition | NM_010118.1 | 2.82862 |
| 6          | Dnajb1      | DnaJ (Hsp40) homolog, subfamily B, member 1 | NM_018808.1 | 2.78017 |
| 7          | Dusp1       | dual specificity phosphatase 1 | NM_013642.2 | 2.533 |
| 8          | Sox9        | SRY-box containing gene 9 | NM_011448.2 | 2.37421 |
| 9          | Zlp36       | zinc finger protein 36 | NM_011756.4 | 2.33651 |
| 10         | Msfd11      | no definition | AK007898 | 2.31434 |
| 11         | Hspb1       | no definition | NM_013560 | 2.30989 |
| 12         | Jun         | Jun oncogene | NM_010591.1 | 2.28214 |
| 13         | Ddit4       | DNA-damage-inducible transcript 4 | NM_029083.1 | 2.25327 |
| 14         | Vegfa       | vascular endothelial growth factor A (Vegfa), transcript variant 1 | NM_001025250.2 | 2.19637 |
| 15         | Herpud1     | homocysteine-inducible, ER stress-inducible, ubiquitin-like domain member 1 | NM_022331.1 | 2.17913 |
| 16         | Ddit3       | DNA-damage inducible transcript 3 | NM_007837.2 | 2.16334 |
| 17         | Mdm2        | transformed mouse 3T3 cell double minute 2 | NM_010786.3 | 2.11273 |
| 18         | Chac1       | ChaC, cation transport regulator-like 1 | NM_026929.3 | 2.08317 |
| 19         | Arc         | activity regulated cytoskeletal-associated protein | NM_018790.2 | 1.99046 |
| 20         | Dnajb9      | DnaJ (Hsp40) homolog, subfamily B, member 9 | NM_013760.4 | 1.961 |
| 21         | Zlnd2a      | zinc finger, AN1-type domain 2A | NM_133349.2 | 1.8778 |
| 22         | Hes1        | hairy and enhancer of split 1 | NM_008235.2 | 1.85536 |
| 23         | Bag3        | BCL2-associated athanogene 3 | NM_013863.4 | 1.85303 |
| 24         | LOC100048331 | PREDICTED: similar to DnaJ (Hsp40) homolog, subfamily A, member 4 | XR_034509.1 | 1.82115 |
| 25         | Hmox1       | heme oxygenase | NM_010442.1 | 1.82111 |
| 26         | Hspa5       | heat shock protein 5 | NM_022310.2 | 1.8205 |
| 27         | Dix2        | distal-less homeobox 2 | NM_010054.1 | 1.62035 |
| 28         | 6430590l03Rik | no definition | XM_489535 | 1.61804 |
| 29         | Junb        | Jun-B oncogene (Junb) | NM_008416.1 | 1.61245 |
| 30         | LOC381140    | no definition | XM_355056.1 | 1.57312 |
| 31         | 5430411C19Rik | PREDICTED: RIKEN cDNA 5430411C19 gene | XM_001478639.1 | 1.56805 |
| 32         | Hspa1a      | no definition | NM_010479 | 1.56028 |
| 33         | Csnp1       | AXIN1 up-regulated 1 | NM_153287.3 | 1.46632 |
| Sort Order | Gene Symbol | Definition                                                                 | Accession         | Log₂ fold change R3 vs. Ctrl |
|------------|-------------|-----------------------------------------------------------------------------|-------------------|-------------------------------|
| 34         | Tnfaip3     | tumor necrosis factor, alpha-induced protein 3                              | NM_009397.2       | 1.45772                       |
| 35         | LOC100048105| PREDICTED: similar to Ubc protein, transcript variant 1                     | XM_001479832.1    | 1.45617                       |
| 36         | Bhlhe40     | basic helix-loop-helix domain containing, class B2                         | NM_011498.4       | 1.39137                       |
| 37         | Dyrk3       | dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3            | NM_145508.2       | 1.3612                        |
| 38         | Egr1        | early growth response 1                                                     | NM_007913.5       | 1.35873                       |
| 39         | Klf9        | PREDICTED: RIKEN cDNA 2310051E17 gene                                        | XM_001479552.1    | 1.35306                       |
| 40         | Snai1       | snail homolog 1                                                             | NM_011427.2       | 1.35105                       |
| 41         | Dusp2       | dual specificity phosphatase 2                                              | NM_010090.2       | 1.34955                       |
| 42         | Ubg         | no definition                                                               | no accession      | 1.3258                        |
| 43         | BC022687    | cDNA sequence BC022687                                                     | NM_145450.3       | 1.31366                       |
| 44         | Btg1        | B-cell translocation gene 1, anti-proliferative                             | NM_007569.1       | 1.2996                        |
| 45         | LOC100046232| PREDICTED: similar to NFIL3/E4BP4 transcription factor                      | XM_001475817.1    | 1.27509                       |
| 46         | HspH1       | no definition                                                               | NM_178187.3       | 1.26359                       |
| 47         | Hst1h2ae    | histone cluster 1, H2ae                                                     | NM_011427.2       | 1.24277                       |
| 48         | mtDNA_ND4L  | no definition                                                               | no accession      | 1.22027                       |
| 49         | Dnajb4      | Dnaj (Hsp40) homolog, subfamily B, member 4                                 | NM_010637.1       | 1.21047                       |
| 50         | Klf4        | Kruppel-like factor 4                                                       | NM_009372.2       | 1.22645                       |
| 51         | Tgif1       | TGFB-induced factor homeobox 1                                              | NM_011800.2       | 1.22207                       |
| 52         | Klf6        | Kruppel-like factor 6                                                       | NM_011800.2       | 1.21047                       |
| 53         | Ppp1r10     | protein phosphatase 1, regulatory subunit 10                               | NM_178187.3       | 1.20916                       |
| 54         | Gm16516     | no definition                                                               | NM_178187.3       | 1.19232                       |
| 55         | Ifrd1       | interferon-related developmental regulator 1                               | NM_178187.3       | 1.18765                       |
| 56         | Slc23a3     | solute carrier family 23 (nucleobase transporters), member 3                | NM_143333.3       | 1.16606                       |
| 57         | Mfsd11      | major facilitator superfamily domain containing 1                           | NM_143333.3       | 1.16498                       |
| 58         | Gm4589      | PREDICTED: hypothetical protein LOC100045678                                | XM_001475512.1    | 1.12553                       |
| 59         | Klf9        | Kruppel-like factor 9                                                       | NM_009372.2       | 1.11181                       |
| 60         | Siah2       | seven in absentia 2                                                         | NM_009372.2       | 1.10454                       |
| 61         | Map1 lc3b   | microtubule-associated protein 1 light chain 3 beta                         | NM_010637.1       | 1.05963                       |
| 62         | Ptk2        | polo-like kinase 2                                                           | NM_010637.1       | 1.05538                       |
| 63         | Fgf21       | fibroblast growth factor 2                                                  | NM_010637.1       | 1.04488                       |
| 64         | Id4         | inhibitor of DNA binding 4                                                  | NM_010637.1       | 1.03533                       |
| 65         | Bbc3        | BCL2 binding component 3 (Bbc3)                                             | NM_010637.1       | 1.03288                       |
| 66         | 6230400G14Rik| no definition                                                               | no accession      | 1.02327                       |
Table 2. List of top 109 up-regulated genes at 6th hour (R6) of anastasis, with log₂ fold change >0.93, compared with Ctrl (untreated cells).

| Sort Order | Gene Symbol | Definition | Accession    | Log₂ fold change R6 vs. Ctrl |
|------------|-------------|------------|--------------|-----------------------------|
| 1          | Inhba       | inhibin beta-A | NM_008380.1 | 3.86584                     |
| 2          | Mmp10       | matrix metallopeptidase 10 | NM_019471.2 | 3.39644                     |
| 3          | Lce1f       | late cornified envelope 1F | NM_026394.2 | 2.99957                     |
| 4          | Serpinb2    | serine (or cysteine) peptidase inhibitor, clade B, member 2 | NM_011111.3 | 2.77022                     |
| 5          | Serpina3h   | serine (or cysteine) peptidase inhibitor, clade A, member 3H | NM_001034870.2 | 2.65107                     |
| 6          | Mmp13       | matrix metallopeptidase 13 | NM_008607.1 | 2.62637                     |
| 7          | Ptnr22      | protein tyrosine phosphatase, non-receptor type 22 | NM_008979.1 | 2.45292                     |
| 8          | Rgs16       | regulator of G-protein signaling 16 | NM_011267.2 | 2.18647                     |
| 9          | Nppb        | natriuretic peptide precursor type B | NM_008726.3 | 2.15071                     |
| 10         | Has1        | hyaluronan synthase1 | NM_008215.1 | 2.14235                     |
| 11         | Dusp5       | no definition | XM_140740.3 | 2.09536                     |
| 12         | Sqstm1      | sequestosome 1 | NM_011018.2 | 2.07477                     |
| 13         | Nupr1       | nuclear protein 1 | NM_019738.1 | 2.06313                     |
| 14         | Sphk1       | sphingosine kinase 1 (Sphk1), transcript variant 2 | NM_025367.5 | 1.94856                     |
| 15         | Dusp4       | dual specificity phosphatase 4 | NM_176933.4 | 1.85742                     |
| 16         | Klhl21      | kelch-like 21 | NM_001033352.3 | 1.84531               |
| 17         | Lor         | loricrin | NM_008508.2 | 1.81763                     |
| 18         | Ndrg1       | N-myc downstream regulated gene 1 | NM_008681.2 | 1.79158                     |
| 19         | Srxn1       | sulfiredoxin 1 homolog | NM_029688.4 | 1.78335                     |
| 20         | Hk2         | PREDICTED: hypothetical protein LOC100047934 | XM_00478074.1 | 1.7519                     |
| 21         | Txnrd1      | thioredoxin reductase 1 (Txnrd1), transcript variant 1 | NM_001042523.1 | 1.75148                     |
| 22         | Angptl4     | no definition | NM_020581 | 1.72982                     |
| 23         | Trib3       | tribbles homolog 3 | NM_175093.2 | 1.72246                     |
| 24         | C330006P03Rik | no definition | no accession | 1.71297                     |
| 25         | Cdkn1a      | cyclin-dependent kinase inhibitor 1A | NM_007669.2 | 1.69118                     |
| 26         | Gdf15       | growth differentiation factor 15 | NM_011819.1 | 1.67887                     |
| 27         | Prkg2       | protein kinase, cGMP-dependent, type II | NM_008926.3 | 1.67374                     |
| 28         | H2afj       | H2A histone family, member J | NM_177688.2 | 1.64825                     |
| 29         | Hbegf       | heparin-binding EGF-like growth factor | NM_010415.1 | 1.61893                     |
| 30         | Trp53inp1   | transformation related protein 53 inducible nuclear protein 1 | NM_021897.1 | 1.61348                     |
| 31         | Glft2       | glutamine fructose-6-phosphate transaminase 2 | NM_013529.2 | 1.58159                     |
| 32         | Slc7a11     | no definition | AK037490 | 1.57761                     |
| 33         | Ndr1        | no definition | NM_008681 | 1.5652                      |
| 34         | Gprc5a      | G protein-coupled receptor, family C, group 5, member A | NM_181444.3 | 1.51339                     |
| 35         | Ibrdc3      | no definition | XM_204030 | 1.49816                     |
| 36         | Ngf         | nerve growth factor, beta | NM_013609.1 | 1.48619                     |
| 37         | Lce1d       | late cornified envelope 1D | NM_027137.2 | 1.44977                     |
| Sort Order | Gene Symbol | Definition | Accession       | Log2 fold change R6 vs. Ctrl |
|------------|-------------|------------|-----------------|-----------------------------|
| 38         | Tpsab1      | tryptase alpha/beta 1 | NM_031187.2     | 1.44267                     |
| 39         | Trhr2b      | 5-hydroxytryptamine (serotonin) receptor 2B | NM_008311.2     | 1.43265                     |
| 40         | Sox4        | SRY-box containing gene 4 | NM_009238.2     | 1.41763                     |
| 41         | Il1r1       | interleukin 1 receptor-like 1 (Il1r1), transcript variant 1 | NM_01025602.1   | 1.3994                      |
| 42         | Prtr9       | RIKEN cDNA A030004J04 gene (A030004J04Rik) | NM_175424.3     | 1.36416                     |
| 43         | Vgf         | VGF nerve growth factor inducible | NM_01039385.1   | 1.35246                     |
| 44         | Errf1       | ERBB receptor feedback inhibitor 1 | NM_133753.1     | 1.34582                     |
| 45         | Il6         | interleukin 6 | NM_031168.1     | 1.33283                     |
| 46         | Gprc5a      | no definition | NM_181444       | 1.31955                     |
| 47         | Antxr2      | anthrax toxin receptor 2 | NM_133738.1     | 1.30719                     |
| 48         | Tgif1       | TGFB-induced factor homeobox 1 | NM_009372.2     | 1.29814                     |
| 49         | Keratin 8   | keratin 8 | NM_031170.2     | 1.28819                     |
| 50         | 2300009A05Rik | PREDICTED: RIKEN cDNA 2300009A05 gene, transcript variant 3 | XM_898537.2     | 1.26684                     |
| 51         | Dppa5a      | developmental pluripotency associated 5 | NM_025274.1     | 1.258                       |
| 52         | Mt2         | metallothionein 2 | NM_008630.2     | 1.2441                      |
| 53         | Plaur       | plasminogen activator, urokinase receptor | NM_011113.3     | 1.22553                     |
| 54         | Thbd        | thrombomodulin | NM_009378.2     | 1.22252                     |
| 55         | LOC100047353 | PREDICTED: similar to myocardial vascular inhibition factor | XM_001477963.1 | 1.22053                     |
| 56         | Csf2        | colony stimulating factor 2 (granulocyte-macrophage) | NM_009369.4     | 1.22019                     |
| 57         | Mep2k1      | mitogen-activated protein kinase kinase 1 | NM_008927.3     | 1.21788                     |
| 58         | Dpp7        | dipeptidylpeptidase 7 | NM_031843.2     | 1.21624                     |
| 59         | LOC672274   | PREDICTED: similar to Transcription factor SOX-4 | XR_003788.1     | 1.21149                     |
| 60         | Bicap       | bladder cancer associated protein homolog | NM_016916.3     | 1.21046                     |
| 61         | Zic3h1      | no definition | NM_01033261.2   | 1.20585                     |
| 62         | Dusp6       | dual specificity phosphatase 6 | NM_026268.1     | 1.20441                     |
| 63         | Areg        | amphiregulin | NM_009704.3     | 1.19656                     |
| 64         | C630022N07Rik | no definition | no accession    | 1.19569                     |
| 65         | Dnmt3l      | DNA (cytosine-5-)methyltransferase 3-like (Dnmt3l), transcript variant 2 | NM_001081695.1 | 1.13992                     |
| 66         | Slc3a2      | solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2 | NM_008577.3     | 1.18244                     |
| 67         | Ern1        | endoplasmic reticulum (ER) to nucleus signalling 1 | NM_023913.2     | 1.15145                     |
| 68         | Dnmt3i      | DNA (cytosine-5-)methyltransferase 3-like (Dnmt3i), transcript variant 2 | NM_001081695.1 | 1.13992                     |
| 69         | D130007C19Rik | no definition | AK051152        | 1.13724                     |
| 70         | LOC100046401 | PREDICTED: similar to SDR2 | XR_0032583.1    | 1.1332                      |
| 71         | Sh3bp2      | SH3-domain binding protein 2 | NM_011893.2     | 1.11999                     |
| 72         | Tgoln1      | trans-golgi network protein | NM_009443.3     | 1.11454                     |
| 73         | Gm12226     | similar to oxidative stress responsive 1 (Rp23-297f14.5) | NM_001099322.1  | 1.11231                     |
| 74         | Stk40       | no definition | NM_028800       | 1.11149                     |
| 75         | Marcks1     | MARCKS-like 1 (Marcks1), mRNA. | NM_010807.3     | 1.09791                     |
| 76         | Ypel5       | yippee-like 5 (Drosophila) (Ypel5), mRNA. | NM_027166.3     | 1.08882                     |
| 77         | Fam180a     | No definition | NM_173375       | 1.08779                     |
| Sort Order | Gene Symbol | Definition                                                                 | Accession   | Log, fold change R6 vs. Ctrl |
|------------|-------------|----------------------------------------------------------------------------|-------------|-----------------------------|
| 78         | Creb3l2     | cAMP responsive element binding protein 3-like 2 (Creb3l2), mRNA.         | NM_178661.3 | 1.08689                     |
| 79         | Ly96        | lymphocyte antigen 96 (Ly96), mRNA.                                       | NM_016923.1 | 1.06285                     |
| 80         | Igf2bp2     | insulin-like growth factor 2 mRNA binding protein 2 (Igf2bp2), mRNA.      | NM_183029.1 | 1.06145                     |
| 81         | Mafg        | v-maf musculoaponeurotic fibrosarcoma oncogene family, protein G           | NM_010756.3 | 1.05594                     |
| 82         | Ctnnbp2nl   | No definition                                                             | NM_030249   | 1.04697                     |
| 83         | Col20a1     | PREDICTED: collagen, type XX, alpha 1 (Col20a1), mRNA.                    | XM_181390.5 | 1.04143                     |
| 84         | Vps37b      | vacuolar protein sorting 37B (yeast) (Vps37b), mRNA.                      | NM_177876.4 | 1.03812                     |
| 85         | A530046M15  | No definition                                                             | XM_488663   | 1.03773                     |
| 86         | Eid3        | EP300 interacting inhibitor of differentiation 3 (Eid3), mRNA.             | NM_025499.2 | 1.03567                     |
| 87         | Nabp1       | oligonucleotide/oligosaccharide-binding fold containing 2A (Oblc2a), mRNA. | NM_028696.2 | 1.0351                      |
| 88         | Pqic1       | PQ loop repeat containing 1 (Pqic1), mRNA.                                | NM_025861.2 | 1.03363                     |
| 89         | Whrn        | whirin (Whrn), transcript variant 6, mRNA.                                | NM_001008795.1 | 1.0255                     |
| 90         | Cish        | cytokine inducible SH2-containing protein (Cish), mRNA.                    | NM_009895.3 | 1.02328                     |
| 91         | Ptpre       | protein tyrosine phosphatase, receptor type, E (Ptpre), mRNA.             | NM_011212.2 | 1.01915                     |
| 92         | Bach1       | BTB and CNC homology 1 (Bach1), mRNA.                                     | NM_007520.2 | 1.01808                     |
| 93         | Cyb5r1      | cytochrome b5 reductase 1 (Cyb5r1), mRNA.                                 | NM_028057.2 | 1.01401                     |
| 94         | Slc1a4      | solute carrier family 1 (glutamate/neural amino acid transporter), member 4 | NM_018861.2 | 1.00471                     |
| 95         | Mnd         | no definition                                                             | AK033889    | 0.998067                    |
| 96         | Slc6a9      | solute carrier family 6 (neurotransmitter transporter, glycine), member 9 | NM_008135.4 | 0.994683                    |
| 97         | LOC100047963| PREDICTED: similar to ADIR1                                                | XM_001479238.1 | 0.994667                    |
| 98         | Atf4        | activating transcription factor 4                                         | NM_009716.2 | 0.982833                    |
| 99         | Ctnnbp2nl   | CTNNBP2 N-terminal like                                                    | NM_030249.3 | 0.970113                    |
| 100        | Mmp9        | matrix metalloproteinase 9                                                | NM_013599.2 | 0.968853                    |
| 101        | Hmgc1       | high mobility group AT-hook 1                                             | NM_016660.2 | 0.96846                     |
| 102        | Phida1      | pleckstrin homology-like domain, family A, member 1                       | NM_009344.1 | 0.963867                    |
| 103        | Aars        | alanyl-tRNA synthetase                                                     | NM_146217.3 | 0.962397                    |
| 104        | Angpt2      | angiopoietin 2                                                            | NM_007426.3 | 0.95926                     |
| 105        | Zswim4      | zinc finger, SWIM domain containing 4                                     | NM_172503.3 | 0.957373                    |
| 106        | Selk        | no definition                                                             | NM_019979.1 | 0.954917                    |
| 107        | Abhd2       | abhydrolase domain containing 2                                           | NM_018111.6 | 0.954587                    |
| 108        | Krtap4-16   | predicted gene, OTTMUSG00000002196                                        | NM_001013823.1 | 0.95438                     |
| 109        | Atg12       | autophagy-related 12                                                      | NM_026217.1 | 0.94998                     |
Table 3. List of top 50 down-regulated genes at 6th hour (R6) of anastasis, with log₂ fold change <-0.95, compared with Ctrl (untreated cells).

| Sort Order | Gene Symbol | Definition | Accession          | Log₂ fold change R6 vs. Ctrl |
|------------|-------------|------------|--------------------|-----------------------------|
| 1          | Hist1h2ak   | histone cluster 1, H2ak | NM_179183.1         | -1.91761                    |
| 2          | Hist1h2ag   | histone cluster 1, H2ag | NM_178186.2         | -1.76767                    |
| 3          | Hist1h2ap   | histone cluster 1, H2ao | NM_179185.1         | -1.7396                     |
| 4          | Hist1h2af   | histone cluster 1, H2af | NM_175661.1         | -1.6854                     |
| 5          | Hist2h2ac   | histone cluster 2, H2ac | NM_175662.1         | -1.6272                     |
| 6          | Slc1a3      | solute carrier family 1 (glial high affinity glutamate transporter), member 3 | NM_148938.2         | -1.61827                    |
| 7          | 9930013L23Rik | no definition | AK018112            | -1.59264                    |
| 8          | Hist1h2ah   | histone cluster 1, H2ah | NM_175659.1         | -1.57002                    |
| 9          | Hist1h2al   | PREDICTED: predicted gene, EG667728 | XP_0035278.1        | -1.56907                    |
| 10         | Hist1h2ad   | histone cluster 1, H2ad | NM_178188.3         | -1.56233                    |
| 11         | Scel        | sciellin    | NM_178184.1         | -1.48845                    |
| 12         | Hist1h2ai   | histone cluster 1, H2ai | NM_178182.1         | -1.40187                    |
| 13         | Fzd2        | frizzled homolog 2 | NM_020510.2         | -1.38033                    |
| 14         | Sdpr        | serum deprivation response | NM_138741.1         | -1.32418                    |
| 15         | Hs3st1      | heparan sulfate (glucosamine) 3-O-sulfotransferase 1 | NM_010474.1         | -1.30977                    |
| 16         | Hist2h2ab   | histone cluster 2, H2ab | NM_179213.3         | -1.30977                    |
| 17         | Kif2c       | kinesin family member 2C (Kif2c) XM_986361 | NM_134471.3         | -1.21821                    |
| 18         | Fam198b     | RIKEN cDNA 1110032E23 gene (1110032E23Rik) | NM_133187.2         | -1.1988                     |
| 19         | Cdc42ep2    | CDC42 effector protein (Rho GTPase binding) 2 | NM_026772.1         | -1.19681                    |
| 20         | Lurap1l     | DNA segment, Chr 4, Brigham & Women’s Genetics 0951 expressed (D4Bwg0951e) | NM_026821.4         | -1.18656                    |
| 21         | Medag       | RIKEN cDNA 6330406l15 gene | NM_027519.1         | -1.18243                    |
| 22         | Disp1       | dispatched homolog 1 | NM_026866.2         | -1.18107                    |
| 23         | Bmp4        | bone morphogenetic protein 4 | NM_007554.2         | -1.16637                    |
| 24         | Rab27a      | RAB27A, member RAS oncogene family | NM_023635.4         | -1.13917                    |
| 25         | Aurka       | aurora kinase A | NM_011497.3         | -1.12507                    |
| 26         | Ncaph       | non-SMC condensin I complex, subunit H | NM_144818.1         | -1.12132                    |
| 27         | Fign1       | fidgetin-like 1 | NM_021891.2         | -1.10521                    |
| 28         | Dbp         | D site albumin promoter binding protein | NM_016974.1         | -1.09945                    |
| 29         | Meis2       | Meis homeobox 2 (Meis2), transcript variant 2 | NM_010825.2         | -1.08487                    |
| 30         | Synpo       | PREDICTED: synaptopodin, transcript variant 2 | XM_981156.1         | -1.08076                    |
| 31         | Hist1h2an   | histone cluster 1, H2an | NM_179184.1         | -1.0804                     |
| 32         | Fam111a     | RIKEN cDNA 4632417K18 gene (4632417K18Rik) | NM_026640.2         | -1.07617                    |
| 33         | Aurkb       | aurora kinase B | NM_011496.1         | -1.07507                    |
| 34         | Anln        | anillin, actin binding protein | NM_028390.2         | -1.07218                    |
| 35         | Tuft1       | tuftelin 1 | NM_011656.2         | -1.06969                    |
| 36         | Cxcl12      | chemokine (C-X-C motif) ligand 12 (Cxc12), transcript variant 1 | NM_021704.2         | -1.0664                     |
| 37         | Sipa1l1     | signal-induced proliferation-associated 1 like 1 | NM_172579.1         | -1.03567                    |
| 38         | Rbms2       | RNA binding motif, single stranded interacting protein 2 | NM_019711.2         | -1.03096                    |
| 39         | Wdr6        | WD repeat domain 6 | NM_031392.2         | -1.02705                    |
Table 4. List of top 15 up-regulated genes during apoptosis (R0) and anastasis (R3 and R6), with log₂ fold change >1 either on R0, R3, or R6, compared with Ctrl (untreated cells).

| Sort Order | Gene Symbol | Definition | Accession      | Log fold change R6 vs. Ctrl |
|------------|-------------|------------|----------------|-----------------------------|
| 40         | Tk1         | thymidine kinase 1 | NM_009387.1 | -1.02669                    |
| 41         | Mylk        | myosin, light polypeptide kinase | NM_139300.3 | -1.01621                    |
| 42         | Slc9a3r1    | solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1 | NM_012030.2 | -1.0137                     |
| 43         | Kif22       | kinesin family member 22 | NM_145588.1 | -1.01346                    |
| 44         | Spef3       | spermatogenesis associated glutamate (E)-rich protein 3 | NM_027650.2 | -1.01229                    |
| 45         | Mrgrf       | MAS-related GPR, member F | NM_145379.2 | -1.01038                    |
| 46         | Bub1b       | budding uninhibited by benzimidazoles 1 homolog, beta | NM_009773.1 | -1.00547                    |
| 47         | Pcgf5       | polycomb group ring finger 5 | NM_029508.3 | -1.00513                    |
| 48         | Marcks      | myriostoylated alanine rich protein kinase C substrate | NM_008538.2 | -0.973133                   |
| 49         | Fam83d      | 2310007D09Rik | NM_027975.1 | -0.966323                   |
| 50         | Slc16a4     | solute carrier family 16 (monocarboxylic acid transporters), member 4 | NM_146136.1 | -0.96461                    |
Figure 4. Change of gene expressions during reversal of apoptosis in human liver cancer HepG2 cells. (A) Confocal and differential interference contrast (DIC) microscopy of untreated liver cells (ii Untreated), cells that were exposed to 4.5% ethanol for 5 hours (ii Treated), and the treated cells that were washed to remove apoptosis inducer and further cultured for 6 hours (iii Washed). Merged images, mitochondria (red) and nuclei (blue) were visualized by confocal microscopy and cell morphology by DIC. Monochrome images, nucleus of the corresponding cells. Scale bar, 10 μm. (B) Quantification of the apoptotic response and its reversal on HepG2 cells. Percentage of the untreated cells, the treated cells (4.5% ethanol, 5 hours) and the washed cells (24 hours) showing mitochondrial fragmentation, nuclear condensation, cell shrinkage, and formation of micronuclei. (C) RT-PCR gel analysis of changes in mRNA levels of ANGPTL4, ATF3, ATG12, CDKN1A, FOS, GUSB, HSPA1B, JUN, MDM2, MMP10 and SOX9 on the untreated (Ctrl), the treated (R0, 4.5% ethanol for 5 hours), and the treated cells that were then washed and incubated in fresh medium for 1 hour (R1), 2 hours (R2), 3 hours (R3), 4 hours (R4), 6 hours (R6), 9 hours (R9), 12 hours (R12), and 24 hours (R24). GUSB serves as housekeeping gene. Sequences of primer sets for detecting targeted genes are available in Table 5.

Table 5. List of primer sequences for RT-PCR.

| Gene   | Accession number | Forward primer | Reverse primer | Amplicon |
|--------|------------------|----------------|----------------|----------|
| ANGPTL4| NM_139314.2      | gacaagaactgcgccaga | gccgttgagttggaatg | 72       |
| ATF3   | NM_001674.3      | cgtgagtcctcggtgctc | gcctgggtgttgaagcat | 112      |
| ATG12  | NM_004707.3      | ttctccgctgcagtttcc | gtctccacagcttttagca | 87       |
| CDKN1A | NM_000389.4      | tggggtgtaccctcttgga | tgaatttcatcaacgccttg | 65       |
| FOS    | NM_005252        | ctggcgctgtgaagacct | cctttttccttctctggaat | 95       |
| GUSB   | NM_000181.3      | cgccctgcctatctgtatcc | tccccacagggagtgttag | 91       |
| HSPA1B | NM_005346.4      | gggtcagggccctaccatt | caaacattccacccaagacaa | 77       |
| JUN    | NM_002228.3      | ccccaagtagatgcgcatgtttt | cgtgccctctcactgcaac | 62       |
| MDM2   | NM_002392.5      | tctgataagttctctcttggt | tgtctcattcacccacatcaaa | 137      |
| MMP10  | NM_002425.2      | gctattgagccctttcttc | cagggtatgggtgccttttg | 147      |
| SOX9   | NM_000346.3      | gtacggcgacgtgcaacaac | ttcgcgcctcgcagaagtc | 74       |
Figure 5. Interaction network of the up-regulated genes during anastasis. The 33 up-regulated genes during anastasis were selected for analysis using GeneMANIA.

Figure 6. Up-regulation of genes and potential corresponding pathways during reversal of apoptosis.
could protect dying cells from free radicals that are generated during apoptosis. Notably, the expression of Bbc3, a pro-apoptotic BH3-only gene to encode PUMA (p53 upregulated modulator of apoptosis) peaks at anastasis (R3–R6), suggesting the sign of anastasis vs apoptosis in the recovering cells during the early stage of the cell recovery process.

To reverse apoptosis, the recovering cells need to remove or recycle the destroyed cellular components, such as the toxic or damaged proteins that are cleaved by caspases, and dysfunctional organelles like the permeabilized mitochondria. Autophagy could contribute to anastasis, as the recovering cells display up-regulation of Atg12 (Figure 3B, Table 2), which is important to the formation of autophagosome to engulf the materials that are then transported to lysosomes or vacuoles for degradation,Sqstm1, which encodes sequestosome and is up-regulated at R6, could play important role in mediating autophagy and DNA damage response during anastasis. In fact, recently studies reveal that autophagy can be activated by the DNA damage response, and play a role in maintaining the nuclear and mitochondrial genomic integrity through DNA repair and removal of micronuclei and damaged nuclear parts. This could suppress mutagenesis and oncogenic transformation to occur in the cells that reverse apoptosis as we have observed after DNA damage, reduction of histone modification, which plays critical role in transcription, DNA replication and repairing. At the late stage of anastasis (R6), various histone genes display significant changes in expression (Table 2, Table 3), suggesting potential connection between histone modification and reversal of apoptosis. Interestingly, significant number of histone genes are down-regulated during anastasis (Table 3). Recent study reported histone degradation in response to DNA damage, and that is important for DNA repair. As dying cells can reverse apoptosis after DNA damage, reduction of histone gene expression could represent the DNA damage response during anastasis.

Arresting cell cycle during anastasis is important as it can allow damaged cells to be repaired before they restore proliferation. This hypothesis is supported by our microarray data that reveals up-regulation of genes that suppress cell cycle (Figure 3A–C). For example, B-cell translocation gene 1 (Btg1) is an anti-proliferative gene, which is up-regulated during the early anastasis (R3). At later stage of anastasis (R6), other cell cycle inhibitors express, including Cdkn1a which encodes p21 that induces cell cycle arrest and senescence, and also Trp53inp1 which encodes tumor protein p53-inducible nuclear protein 1 that can arrest cell cycle independent to p53 expression. These suggest that cell cycle is suppressed by multiple pathways during anastasis.

We also identified genes that are up-regulated both during apoptosis and anastasis, such as Gadd45g, and Rnu6 (Figure 3C, Figure 4). Gadd45g functions in growth arrest and DNA repair, and therefore, could be the cytoprotective mechanism that preserves DNA in the dying cells during cell death induction (R0), and promotes the injured cells to repair when the environment is improved (R3 and R6). Rnu6 encodes U6 small nuclear RNA, which is important for splicing of a mammalian pre-mRNA. Upregulation of Rnu6 from R0 to R6 suggests that post-transcriptional regulation could be involved during apoptosis and anastasis. In fact, translational regulation also contributes to anastasis. For example, caspase-3, PARP and ICAD are cleaved in dying cells during apoptosis, and the non-cleaved form of corresponding proteins restores after anastasis (Figure 1B). Interestingly, the mRNA level of caspase-3, PARP and ICAD did not show significant increase during and after anastasis (see Data availability). This suggests the contribution of translational regulation during anastasis.

Our study provides new insights into the mechanisms and consequences of anastasis (Figure 6). Researchers can analyse our microarray data to further identify the hallmarks of anastasis, understand its role, elucidate molecular mechanisms that reverse apoptosis, and develop therapeutic strategies by controlling anastasis. To identify the genes that display specific change on a transcriptional level, software such as Spotfire can be used to view the gene expression pattern at different time points during the reversal of
apoptosis. To study the molecular mechanism of anastasis, Ingenuity Pathway Analysis can be used to create mechanistic hypotheses according to the transcriptional profile. To identify drugs that modulate anastasis, Connectivity Map can be used to identify small molecules that promote or suppress anastasis based on its gene expression signature. Anastasis could be a cell survival phenomenon mediated by multiple pathways, so by comparing the gene expression profiles, researchers can study its potential connection to other cellular processes, such as anti-apoptotic pathways, autophagy, and stress-inducible responses. By searching the molecular signature of anastasis, researchers can study its potential contribution to physiological and pathological conditions, such as recovery from heart failure, wound healing, mutagenesis, tumour evolution, cancer recurrence and metastasis. Further data analysis will stimulate the generation of hypotheses for future studies involving anastasis. As our understanding of anastasis mechanism expands, it will uncover its potential impacts on physiology and pathology, and offer exciting new therapeutic opportunities to intractable diseases by mediating cell death and survival (Figure 7).

Data availability
Figshare: Raw data for Tang et al., 2016 “Molecular signature of anastasis for reversal of apoptosis” doi: 10.6084/m9.figshare.4502732

Supplementary material
Supplementary Figure 1: Technical validation of microarray data. The three biological replicate samples of microarray data were shown to cluster together by using principal component analysis (PCA).

Supplementary Figure 2: Genes display unique expression patterns at each timepoint. Output of all genes analysed by Spotfire (K cluster, also see Data availability).

Supplementary Figure 3. Uncropped agarose gel images. RT-PCR gel analysis of changes in mRNA levels of ANGPTL4, ATF3, ATG12, CDKN1A, FOS, HSPA1B, JUN, MDM2, MMP10 and SOX9 on untreated (Lane 1: Ctrl), treated (Lane 2: R0, 4.5% ethanol for 5 hours), and the treated cells that were then washed and incubated in fresh medium for 1 hour (Lane 3: R1), 2 hours (Lane 4: R2), 3 hours (Lane 5: R3), 4 hours (Lane 6: R4), 6 hours (Lane 7: R6), 9 hours (Lane 8: R9), 12 hours (Lane 9: R12), and 24 hours (Lane 10: R24). GUSB serves as housekeeping gene. Lane M is loaded with 25 bp DNA Ladder (Invitrogen). Sequences of primer sets for detecting targeted genes are available in Table 5.

Supplementary Figure 4: Increase in mobility of HeLa cells after reversal of ethanol-induced apoptosis. Time-lapse live-cell fluorescence microscopy of HeLa cells before, during, and after exposure to the apoptotic stimulus, ethanol. The same cells before ethanol induction (Untreated), treated with 5% ethanol in culture medium for 55 minutes (Induced, ii to iv), and then washed and further cultured with fresh medium (Washed, v to xx). Green, white and yellow lines indicate the footprints of the corresponding same cells that reversed apoptosis throughout the experiment with time. Cells without displayed hallmarks of apoptosis at late stage, such as cell shrinkage and nucleus condensation (without track), had lower mobility than the tracked cells. Merged images, mitochondria (red) and nuclei (blue) were visualized by fluorescence, and cell morphology by differential interference contrast (DIC) microscopy. Time presented as hour:minute. Scale bar, 10 μm. Adopted images reprinted with permission.

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Current Referee Status:  ✔  ✔  ✔

Leonard K. Kaczmarek
Department of Pharmacology, Yale School of Medicine, New Haven, CT, USA

The data presented build on the authors’ earlier studies of reversal of apoptosis. They provide useful information documenting changes in gene expression following 5 hours of exposure to ethanol, an apoptotic stimulus to primary mouse liver cells. The findings only make sense when taken in combination with the authors previous work, which provides a compelling case that the recovery from apoptosis is indeed occurring, and the manuscript documents some of this by reprinting figure panels from an earlier publication in Figure 1.

My only minor comment is that, at least in some cells such as neurons, cleavage of caspase-3 can be a regulatory signal that does not herald cell death, but may influence biological processes such as synaptic function.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Takafumi Miyamoto
University of Tokyo, Tokyo, Japan

The authors sincerely responded to my comment with deep consideration. Therefore I have no objection to this article being indexed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.
Sanzhen Liu  
Department of Plant Pathology, Kansas State University, Manhattan, KS, USA

The manuscript by Tang et al. was focused on the elucidation of the molecular mechanisms of an important phenomenon, anastasis, through time-course expression profiling. Anastasis was recently discovered and has not been fully studied yet. It's molecular basis remains to be uncovered. The study provided useful information to better understand this underexplored process. Overall, the experiment was well designed. The time course experiment included six time points, untreated samples as the control, toxin-induced apoptosis, and four time points after removal of toxin. Three biological replicates were performed at each time point. Figure 1 illustrated the experimental design very well. The biological interpretation of microarray results is reasonable. The reviewer has no major concerns. However, several minor changes are needed, especially for the presentation of figures, which could be improved.

First, no multiple test correction was mentioned in the microarray analysis section. It was described that the p-value less than 0.05 was used to declare statistical significance. The reviewer would suggest the authors confirm that. A false discovery rate (FDR) method is needed for multiple test correction.

Second, the PCA result from Figure 2A showed that three biological replicates were closely clustered, which showed a good repeatability. However, the goal of PCA is not just check the repeatability of three replicates of each group (time point). PCA can be also used to examine the relationship among groups. My recommendation is that the authors provide more description for the PCA result. In addition, in Figure 2A, the percentage of PC2 explaining total variation was masked. But based on the value of PC3, it should be greater than 5.89%. Given the high value of PC1, I would suggest plotting a two dimension PCA plot to display the result or re-plotting this three-dimension plot.

Third, it would be useful to list the number of significant differential expression for each comparison. And I guess the clustering result in Figure 3 presented all significant genes.

Figure 4 showed some interesting result about gene interactions. I did not see enough description about this figure in the main text.

Editorial comments:
In the Abstract, “whole genome” can be replaced by “genome-wide”.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

**Competing Interests:** No competing interests were disclosed.
This study unravels the gene regulatory network that seems to be involved in the process of anastasis. It is interesting that the authors found various genes that appear to participate in ethanol-induced anastasis, suggesting that the dynamic reconstitution of gene regulatory networks might be a prerequisite for rescuing cells from the brink of cell death. Overall, this work is worth being indexed. However, I would like to see the following points in the research addressed, before approval:

1. Anastasis is a developing concept rather than an established one. It would be better to show the expression dynamics of caspase-3, PARP, and ICAD at all analyzed time points (Cont, R0, R3, R6, R24, and R48). In addition, why don't the authors show apoptotic DNA fragmentation to make sure that all the analyzed cells in the anastasis stage definitely underwent apoptosis?

2. I may have missed noting this, but there is no statistical analysis of the gene expression changes observed in the microarray data. In Fig. 2B, the expression levels of several genes seem different in the same time point replicates. It would be better to show the genes that were induced or suppressed during anastasis, along with the statistical significance of the differences.

3. Given the importance of understanding the mechanism of anastasis, it would be better to verify the data obtained from microarray analysis, by using quantitative PCR or Western blotting.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

**Competing Interests:** No competing interests were disclosed.

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**Author Response 03 Feb 2017**

**Ho Lam Tang**, Johns Hopkins University, USA

We thank for the enthusiasm and valuable input from the reviewer, and have made the following changes:

1. We have included the Western blot data (Figure 1B), which shows that caspase-3, PARP and ICAD were cleaved during apoptosis, but then recovered to their original level at 24 hours after removal of the cell death stimulus. Interestingly, our microarray data shows that their level of mRNA remained no significant change at all time points (3, 6, 24 and 48 hours) after removal of the cell death stimulus, compared with the untreated (control) cells (data available at figshare, please see Data availability in the manuscript), suggesting the recovery of corresponding proteins is contributed by the regulation of translation during and after anastasis. The related data and discussion are included in our revised manuscript.

Our earlier studies using time-lapse live cell microscopy and comet assay demonstrated that the current apoptotic induction (4.5% ethanol, 5 hours) can trigger DNA damage. After removal of the stimulus, major of the dying cells can recover. Interestingly, some cells that reversed apoptosis display chromosomal abnormality and oncogenic transformation, indicating reversibility of apoptosis after DNA damage. In our current study, we further found significant reduction of mRNA level of multiple histone genes during anastasis. Notably, cellular levels of histones reduce in response to DNA damage, as to enhance DNA repairing. Therefore, reduction of expression of histones during anastasis could be a sign of...
cells that recover from DNA damage after apoptosis.

2. We have included supplementary data with corresponding p-value for statistical significance of fold change for all of the 3 biological replicants of each gene (see Data availability). The software for the microarray data analysis is mentioned at the “Materials and methods” section.

3. We have verified our data by RT-PCR in human liver cancer HepG2 cell line, and included the data in the new Figure 4.

**Competing Interests:** No competing interests were disclosed.