Differential Proteome Analysis of Replicative Senescence in Rat Embryo Fibroblasts

Silvia Benvenuti†‡§, Rainer Cramer†‡§, Christopher C. Quinn¶, Jim Bruce||, Marketa Zvelebil†‡§, Steven Corless‡, Jacquelyn Bond‡, Alice Yang‡, Susan Hockfield¶, Alma L. Burlingame†‡§, Michael D. Waterfield†‡§, and Parmjit S. Jat†‡§**

Normal somatic cells undergo a finite number of divisions and then cease dividing whereas cancer cells are able to proliferate indefinitely. To identify the underlying mechanisms that limit the mitotic potential, a two-dimensional differential proteome analysis of replicative senescence in serially passaged rat embryo fibroblasts was undertaken. Triplicate independent two-dimensional gels containing over 1200 spots each were run, curated, and analyzed. This revealed 49 spots whose expression was altered more than 2-fold. Of these, 42 spots yielded positive protein identification by mass spectrometry comprising a variety of cytoskeletal, heat shock, and metabolic proteins, as well as proteins involved in trafficking, differentiation, and protein synthesis, turnover, and modification. These included gelsolin, a candidate tumor suppressor for breast cancer, and α-glucosidase II, a member of the family of glucosidases that includes klotho; a defect in klotho expression in mice results in a syndrome that resembles human aging. Changes in expression of TUC-1, -2, -4, and -4β, members of the TUC family critical for neuronal differentiation, were also identified. Some of the identified changes were also shown to occur in two other models of senescence, premature senescence of REF52 cells and replicative senescence of mouse embryo fibroblasts. The majority of these candidate proteins were unrecognized previously in replicative senescence. They are now implicated in a new role. Molecular & Cellular Proteomics 1:280–292, 2002.

Cancer arises as a consequence of the accumulation of multiple independent mutations in genes that regulate cell proliferation and survival (1). The acquisition of an unlimited proliferative potential has been proposed to be one of the critical steps in this process, because normal cells can only undergo a finite number of divisions when cultured in vitro before undergoing replicative senescence (2). Even though replicative senescence has been studied extensively and can be overcome by immortalizing genes, the underlying molecular basis is still not understood fully.

In human somatic cells telomere shortening is a critical component of the machinery that counts the number of cell divisions and therefore entry into senescence. It was proposed initially that reconstitution of telomerase activity resulting in maintenance of telomeres was sufficient for immortalization of human somatic cells, but others have found that this is not sufficient and requires additional activities such as those that can be provided by the SV40 (simian virus 40) large T antigen or inactivation of the pRB/p16 INK4 pathway (3–5). Further studies have now shown that in freshly isolated human mammary fibroblasts and endothelial cells, reconstituted telomerase activity was sufficient neither for immortalization nor maintenance of the immortal state in cell lines that had been immortalized with a combination of the SV40 T antigen and the catalytic subunit of telomerase (6). Inactivation of SV40 T antigen in these cells resulted in a rapid and irreversible cessation of cell growth and entry into senescence.

Even though telomere shortening cannot be demonstrated in rodent cells, they, too, have a finite life span. In contrast to human cells this can be overcome readily in rodent cells by either the exogenous introduction of any member of the family of viral and cellular immortalizing genes, such as SV40 T antigen, or even by spontaneous mutation. Interestingly like the human cells immortalized with SV40 T antigen and hTERT, rodent cells expressing SV40 T antigen proliferate indefinitely and are absolutely dependent upon its continued expression for maintenance of growth (7). Inactivation also results in a rapid and irreversible cessation of growth and entry into senescence (8). Moreover, we have shown that primary mouse embryo fibroblasts are able to measure their proliferative life span even in the presence of SV40 T antigen at the normal rate (9). Taken together these results have raised the possibility that the non-telomere shortening-dependent regulatory components of the finite proliferative life span may be conserved between human and rodent cells and that human cells may have acquired telomere shortening as a further control mechanism.

Replicative senescence is an asynchronous process...
whereby a growing culture gives rise to an irreversibly arrested culture. The model systems that are commonly used for its study involve the isolation and serial in vitro cultivation of primary fibroblasts. Initially these cells proliferate exponentially but cease dividing after some passages, at which point the cell numbers no longer increase. The loss of proliferative potential in such heterogeneous cultures of primary cells is asynchronous. When these cells have reached the end of their in vitro mitotic lifespan, they can be maintained and remain metabolically active but cannot be induced to undergo new rounds of cell division (10). In such model systems, the culture as a whole divides initially and undergoes growth arrest toward the end; however there can be growth-arrested cells in the early passages and dividing cells toward the later passages.

The senescent phenotype is dominant as fusions of senescent cells with dividing cells give rise to senescent cells (11). Even though a variety of traditional approaches have been utilized to try to identify the underlying changes that are the cause of senescence, this process is still not understood fully, probably because these procedures were insufficient to analyze comprehensively such a complex process. New approaches to address complex biological systems include DNA microarrays that monitor global changes in mRNA expression (12). However studies in Saccharomyces cerevisiae and human liver have suggested that mRNA levels may correlate poorly with corresponding protein levels (13, 14), and mRNA-based assays are unable to detect changes in protein level because of stability and changes in post-translational modifications. Another approach would be to analyze changes in protein expression that have the potential for resolution of all expressed proteins in a cell (proteome), which can then be identified by mass spectrometry.

Even though not all proteins may be resolved by two-dimensional (2-D)\(^1\) gels and also may not be identified by mass spectrometry, we have initiated a differential proteomic approach to study replicative senescence. This was done, because it has the potential for identifying changes in protein expression, post-translational modification, stability, and even changes in cellular localization. Furthermore, we have chosen to study the protein expression profiles of serially passaged rat embryo fibroblasts (REFs), rather than primary human fibroblasts, to minimize differences because of epigenetic variation between cells obtained from different donors and also, because human fibroblasts have much longer finite proliferative lifespan in vitro. Human fibroblasts are capable of undergoing 50–60 divisions before undergoing replicative senescence, in contrast to 20–30 divisions for rodent embryo fibroblasts. The issue of epigenetic variation was critical, because we needed to be able to go back and repeat the passaging with freshly isolated identical cells to prepare protein extracts for validation of the proteome analysis and also extraction of RNA for determining whether changes at the protein level correlate with changes at the RNA level. Cells that are cultivated serially after freezing exhibit an altered finite mitotic life span.

Changes in protein profiles upon cellular senescence were monitored by high resolution 2-D polyacrylamide gel electrophoresis, and differentially expressed protein spots were identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and nano-HPLC electrospray ionization tandem mass spectrometry (ESI-MS/MS). This analysis identified 49 spots whose expression changed more than 2-fold upon replicative senescence; 32 of these spots were up-regulated, 12 were down-regulated, and five displayed an altered migration pattern. The majority of these proteins were unrecognized previously in replicative senescence. They are now implicated in a new role.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—REFs were prepared from 12–13-day-old Sprague-Dawley rat embryos, cultured, and passaged serially. All cultures were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin. REF52 cells obtained from Scott Lowe were propagated in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin. All media and components were obtained from Invitrogen.

2-D Polyacrylamide Gel Electrophoresis—2-D gels were prepared and run as described previously (15). Staining, scanning, and curation of primary images with subsequent analysis to identify differential spots were all carried out by previously published procedures (15).

**Protein Identification by Mass Spectrometry**—Identifications of all differentially expressed proteins utilized a standard approach using MALDI-MS and if necessary ESI-MS/MS. Differential spots were excised from one of the triplicate gels, which showed the highest expression level for each spot. Prior to mass spectrometry, tryptic in-gel digests were carried out on all samples using a protocol similar to already published procedures (e.g., donatello.ucsf.edu/ingel.html; see Ref. 16). Details of the procedures for peptide mass mapping by matrix-assisted laser desorption/ionization mass spectrometry and peptide sequencing by nano-HPLC electrospray ionization tandem mass spectrometry are provided as Supplementary Material.

1-D Western Blotting—Cell lysates for immunoblotting were prepared in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0). Protein concentrations were determined using the Bradford reagent (Bio-Rad). 30 \(\mu\)g of each protein lysate was fractionated on an SDS polyacrylamide gel, transferred to Hybond C membrane, and probed with the following antibodies: \(\alpha\)-glucosidase (StressGene), HSP27 Ab-1 (NeoMarkers), HSP70 W27 (Santa Cruz Biotechnology), CDC47 (NeoMarkers), gelatin (kindly provided by Helen Yin), cyclin A Ab-4 (Oncogene Science), and p19\(^{ARF}\) (Abcam).

**Semiquantitative RT-PCR Analysis**—First strand cDNA was prepared from 2 \(\mu\)g of total RNA using SuperScript\(^{TM}\) II Moloney murine leukemia virus RNaseH\(^{-}\) reverse transcriptase from Invitrogen according to the manufacturer’s instructions. RNA was denatured

---

\(^1\) The abbreviations used are: 2-D, two-dimensional; REF, rat embryo fibroblast; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; HPLC, high pressure liquid chromatography; ESI-MS/MS, electrospray ionization tandem mass spectrometry; RT, reverse transcriptase; HSP, heat shock protein; 1-D, one-dimensional.
(65 °C for 5 min) in the presence of an oligo(dT) primer (0.5 μg; Promega) and dNTPs (0.5 mm; Promega) and quickly chilled on ice. Ribonuclease inhibitor (RNasin; Promega) and dithiothreitol (100 mm) were then added, together with 1× first strand buffer, and incubated at 42 °C for 2 min. Moloney murine leukemia virus RNaseH− reverse transcriptase (200 units) was added, and reactions were incubated at 42 °C for 50 min, followed by heat inactivation at 70 °C for 15 min. For each gene, an optimal cycle number was established that enabled the bands to be visible on a gel but did not result in saturation of the amplification procedure. This was done partially by confirming that the same relative intensities were obtained when the cycle number was increased by two cycles. The sequences of the primers used for PCRs, along with the product size, cycle numbers, and annealing temperatures, are provided as Supplementary Material. All PCR reactions were carried out in 50 μl and contained 1 μl of first strand cDNA. They also contained 0.5 μg of each oligonucleotide primer, 2.5 units of *Thermus aquaticus* (Taq) DNA polymerase (Promega), 1× PCR buffer (10 mM Tris-HCl, pH 9, 50 mM KCl, 0.1% (w/v) Triton X-100 (10× PCR buffer provided by Promega), 0.5 mm dNTPs (Promega), and 1.5–2.5 mm MgCl2 (Promega). The optimum MgCl2 concentration was determined for each primer set and is also provided as Supplementary Material.

A 5-min 95 °C denaturation step was used prior to amplification. The amplification parameters were denaturation at 94 °C for 1 min, annealing at the specific temperature for each primer pair for 1 min, and extension at 72 °C for 1 min, with a final extension of 5 min at 72 °C after the last cycle. For each PCR, appropriate controls were carried out to check for nonspecific amplification. All PCR primers were designed using Primer3 software (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

**Premature Senescence of REF52 Cells—**BOSC23 cells (1.75 × 10⁶) were plated in a 6-cm dish and transfected 24 h later with 3 μg of either pBabePuroEJras or pBabePuro using FuGENE (Roche Molecular Biochemicals). 48 h after transfection, the virus-containing medium was removed, filtered (through a 0.45 μM filter), and used to infect REF52 cells in the presence of 8 μg/ml polybrene (Aldrich). REF52 were plated at 8 × 10⁶ cells per 10-cm dish and incubated overnight prior to infection. For the infection, the culture medium was replaced by 2 ml of virus-containing medium for 2 h. The infection was repeated a second time 6 h later. Sixteen h later, the infected cultures were subjected to 2 μg/ml puromycin selection. The medium was changed on the third day, and the infected cells were extracted with RIPA buffer after 7 days.

**RESULTS AND DISCUSSION**

**2-D Gel Analysis**

REFs were prepared from 12–13-day-old Sprague-Dawley rat embryos and passed using the 3T3 pasaging regime of Todaro and Green (17). Cells were passed serially until they ceased to divide and cell numbers no longer increased, which takes about five passages. Cultures were continually examined microscopically to ensure that there were no mitotic cells visible when passing was ceased. This involved plating 2.6 × 10⁷ cells per 15-cm dish. Cells were plated on day 0 and allowed to adhere, the medium was changed on day 1, and the cells passaged on day 3. For preparation of cell lysates, cells were harvested on day 2. In this way all lysates were prepared from cultures that were in fresh medium and sub-confluent and thus not quiescent. This was done to ensure that only changes in expression because of senescence were being examined.

Triplet independent total protein extracts prepared from three serially passaged independent dishes of REFs at P2 (proliferating cells), P3, P4, and P5 (senescent cells) were fractionated on 2-D gels to visualize changes in the proteome. The fractionated proteins were detected by staining the gels with the fluorescent dye OGT MP17, followed by scanning at a detection level of less than 1 ng of protein. A representative 2-D gel for P2 and P5 senescent REFs is shown in Fig. 1. The triplicate scanned gels were then subjected to triplicate cura-}

### Protein Identification by Mass Spectrometry

**Peptide Mass Mapping by MALDI-MS—**Analysis of the differentially regulated spots by MALDI-MS yielded 24 single positive identifications, two double positive identifications, six single putative identifications, one double putative identification, and one double mixed (positive and putative) identifica-
FIG. 1. A representative 2-D gel image of P2 and P5 cells obtained upon fractionation of 150 μg of total cell extract.
Proteomic Analysis of Replicative Senescence

| Table I  
| Percent homology between the triplicate gels  
| Triplicate 2-D gels were run, curated, and compared.  
| Reference gel | Gel II | Gel III |
|---------------|--------|--------|
| P2            | 100    | 98.6   | 97.1   |
| P3            | 100    | 98.0   | 99.0   |
| P4            | 100    | 95.0   | 93.0   |
| P5            | 100    | 96.0   | 96.1   |

The proteins identified by searching the NCBI protein database only 15 proteins were identified as rat proteins, whereas three were from mouse, one was from chicken, one was from Chinese hamster, and 10 were human. MS/MS data from two spots identified human proteins with some peptides giving matches to the mouse and bovine homologues that did not match the human protein. Similarly, MS/MS data from one other spot resulted in peptide matches not matching the rat protein but rather the Chinese hamster homologue. The chicken myosin heavy chain identified in spot 190 was quite unusual, because a very close rat homologue exists in the NCBI protein database.

The differentially expressed proteins comprised a variety of cytoskeletal, heat shock, and metabolic proteins, as well as proteins involved in trafficking, differentiation, and protein synthesis, turnover, and modification. For the purposes of the discussion below, we have assumed that all proteins identified by mass spectrometry for each spot were expressed differentially. However, this can clearly not be the case for spots that yielded multiple proteins; for these spots Western blot analysis using specific antibodies will be required to determine the identity of the differential protein.

Cytoskeletal Proteins—A number of the proteins identified, like gelsolin, β-tubulin, and Arp2 (actin-related protein 2), have been shown previously to be involved in cytoskeletal scaffolding. Most of these proteins were up-regulated during senescence; however β-tubulin was down-regulated. It is perhaps not surprising that proteins involved in the cytoskeleton show changes in expression upon replicative senescence, because senescent cells are very different morphologically from growing cells; they have the classic fried egg morphology of senescent cells. They are much larger, have lots of stress fibers, and overexpress many extracellular matrix components such as collagenases, collagen, stromolysin, and fibronectin.

Gelsolin is involved in nucleation of actin filaments and is responsible for regulating the growth rate of these filaments and therefore has a role in cell motility (19). It mediates the rapid remodeling of cortical actin filaments and has a role in stress fiber-dependent cell function (20). Gelsolin is also a caspase 3 substrate and is cleaved during Fas-mediated apoptosis (21) and gives rise to a fragment that can depolymerize actin filaments and either promote or inhibit apoptosis depending on the cell type (22).

Here we have found that gelsolin (spot 190) was up-regulated 4.8-fold upon replicative senescence; growing cells have low levels of gelsolin whereas post-mitotic senescent cells have high levels of gelsolin. This was consistent with previous observations that gelsolin was down-regulated in several types of transformed cells and tumors (23–25). The partial or total loss of gelsolin expression in a majority of breast cancers of diverse aetiologies has led to the proposal that gelsolin is a candidate tumor suppressor gene for breast cancer (26). Both gelsolin protein and its mRNA have been shown to be down-regulated in cancer cells, suggesting that sample 504, and heat shock protein 27 in sample 1295. For five previously unidentified spots, positive protein identification was obtained. The comparison of the results from the MALDI-MS and ESI-MS/MS data demonstrated that both techniques show comparable sensitivity in protein identification. Furthermore, this comparison also showed that the criteria applied for protein identification were reliable and gave virtually no false identifications, with a minimal loss in analytical sensitivity.

Description of Differentially Expressed Proteins

Positive protein identifications obtained by MALDI-MS peptide mass mapping were confirmed in all cases where ESI-MS/MS was employed. All putatively identified proteins by MALDI-MS peptide mass mapping were also confirmed by ESI-MS/MS whenever sufficient data were obtained. In addition to these confirmations three more proteins were identified, t-complex polypeptide in sample 497, lamin A or C2 in sample 504, and heat shock protein 27 in sample 1295. For five previously unidentified spots, positive protein identification was obtained. The comparison of the results from the MALDI-MS and ESI-MS/MS data demonstrated that both techniques show comparable sensitivity in protein identification. Furthermore, this comparison also showed that the criteria applied for protein identification were reliable and gave virtually no false identifications, with a minimal loss in analytical sensitivity.
### Table II

Protein annotations

| Up-regulated features | MALDI-MS spot identification | ESI spot identification |
|-----------------------|------------------------------|------------------------|
| ID        | MW          | pI | F.C. | P5 vs. P2 | | |
| 102      | 125386      | 5.48  | +3.2 | | | 2-Oxoglutarate dehydrogenase precursor (h) |
| 104      | 125386      | 6.37  | +2.3 | | | O-GlcNAc transferase p110 subunit (r) |
| 108      | 124240      | 6.53  | >10  | | | α-Glucosidase II, α subunit (m) |
| 110      | 123105      | 5.53  | >10  | | | α-Glucosidase II, α subunit (m) |
| 112      | 113345      | 5.82  | >10  | | | α-Glucosidase II, α subunit (m) |
| 158      | 98305       | 5.01  | +2.3 | | | |
| 177      | 933348      | 6.52  | +2.2 | | | |
| 185      | 91608       | 4.96  | >10  | | | Heat shock protein 90-α (h) |
| 190      | 91608       | 5.66  | +4.8 | | | Gelsolin (h and m) + myosin heavy chain (c) |
| 223      | 83776       | 5.31  | >10  | | | |
| 248      | 81062       | 7.08  | >10  | | | |
| 290      | 84528       | 6.64  | +2.0 | | | |
| 369      | 67881       | 5.90  | >10  | | | |
| 426      | 64712       | 6.19  | +2.9 | | | |
| 493      | 60767       | 7.56  | +2.0 | | | |
| 495      | 60767       | 6.47  | +3.3 | | | |
| 497      | 60462       | 6.06  | +2.0 | | | |
| 504      | 60158       | 6.94  | +2.2 | | | |
| 677      | 47888       | 5.57  | +3.0 | | | |
| 713      | 45110       | 5.14  | +3.0 | | | |
| 720      | 40126       | 5.68  | +2.0 | | | |
| 900      | 40010       | 5.71  | +2.1 | | | |
| 1075     | 47882       | 5.79  | +2.0 | | | |
| 1076     | 33968       | 7.22  | 2.0  | | | |
| 1093     | 23343       | 5.84  | >10  | | | |
| 1095     | 33385       | 6.42  | +2.0 | | | |
| 1124     | 32329       | 5.34  | +2.0 | | | |
| 1264     | 25789       | 6.05  | >10  | | | |
| 1302     | 24177       | 5.58  | +3.0 | | | |
| 1319     | 23370       | 5.57  | >10  | | | |
| 1331     | 158886      | 6.38  | +2.0 | | | |
| 153      | 79874       | 4.76  | +2.5 | | | |
| 278      | 64858       | 5.09  | +3.2 | | | |
| 304      | 63329       | 6.09  | +2.4 | | | |
| 460      | 53263       | 7.39  | +2.0 | | | |
| 736      | 39212       | 6.13  | +2.6 | | | |
| 863      | 34304       | 5.41  | +4.7 | | | |
| 882      | 34172       | 5.57  | +3.9 | | | |
| 1054     | 23301       | 5.01  | +2.0 | | | |
| 1299     | 14189       | 5.66  | +2.2 | | | |
| 1295     | 24425       | 5.87  | +2.0 | | | |
| 1299     | 24459       | 5.78  | +2.1 | | | |

### Down-regulated features

| ID        | MW          | pI | F.C. | P2 vs. P5 | | |
| 15        | 158886      | 6.38  | +2.0 | | | |
| 153       | 79874       | 4.76  | +2.5 | | | |
| 278       | 64858       | 5.09  | +3.2 | | | |
| 304       | 63329       | 6.09  | +2.4 | | | |
| 460       | 53263       | 7.39  | +2.0 | | | |
| 736       | 39212       | 6.13  | +2.6 | | | |
| 863       | 34304       | 5.41  | +4.7 | | | |
| 882       | 34172       | 5.57  | +3.9 | | | |
| 1054      | 23301       | 5.01  | +2.0 | | | |
| 1299      | 14189       | 5.66  | +2.2 | | | |
| 1295      | 24425       | 5.87  | +2.0 | | | |
| 1299      | 24459       | 5.78  | +2.1 | | | |

Features that shift in their migration

| ID        | MW          | pI | F.C. | | |
| 331       | 71199       | 6.67  | | | |
| 332       | 71199       | 6.56  | | | |
| 1117      | 22014       | 6.19  | | | |
| 1348      | 22134       | 6.20  | | | |
| 1351      | 22134       | 6.10  | | | |
Proteomic Analysis of Replicative Senescence

**Heat Shock Proteins**—Four HSPs were identified during this study. HSP105 (spot 110), HSP90α (spot 185), and HSP27 (spot 1302) were up-regulated, and HSP70, identified in spot 153, together with Sec23 and ischemia-responsive 94-kDa protein, was down-regulated. HSPs are a group of proteins that are highly conserved from bacteria to mammals and classified into different families according to their size as follows: HSP110/105, 90, 70, 60, 40, and 27. They were identified initially by virtue of their rapid induction under stress conditions and proposed to play a critical role in protection from hypothermia and other types of stress (32–34). However, they are now known to play essential roles under normal physiological conditions such as assisting the folding of newly synthesized proteins, protein translocation across organelle membranes, promoting assembly or disassembly of oligomeric proteins, and facilitating protein degradation of incorrectly folded or denatured proteins. They have also been shown to localize to the centrosome, but the significance of this localization is not clear (35). The mammalian HSP110/105 may exist as two forms, α and β. Even though both forms are induced upon stress, the α form is also expressed constitutively (36). Both forms associate with HSP70 and have been proposed to regulate negatively its chaperone activity. They are also induced upon adipocyte differentiation (37). Our finding that HSP27 was up-regulated upon senescence is in accordance with the observation that overexpression of HSP27 in bovine pulmonary endothelial cells stimulates their growth rate and accelerates the rate at which the cultures reach senescence (38). HSP27 is also expressed differentially between human breast luminal and myoepithelial cells (15).

The finding that heat shock proteins were expressed differentially may be particularly relevant, because it has been proposed by some that replicative senescence in rodent cells may be caused by the stress of in vitro tissue culture (62, 63). It has been observed that increased stress such as freezing-thawing and growth at lower temperatures can shorten the finite proliferative life span (9). Increases in resistance to stress have been shown to increase chronological lifespan in *Drosophila melanogaster* and *Caenorhabditis elegans* by acting on post-mitotic cells rather than affecting replicative potential (64). Stress resistance could be increased by reducing expression of HSP90, which can down-regulate heat shock transcription factor HS1 (64). Induction of HSP70, the most abundant and most evolutionarily conserved, by stress is significantly lower in late passage senescent fibroblasts, probably because of reduced levels of HSF1 that are required for transcriptional up-regulation of HSP70 (65). Recently two mortalin genes (Mot1 and Mot2), members of the HSP70 family that are derived from two distinct genes, were identified. They encode proteins that differ by only two amino acids but exhibit different subcellular localizations and have contrasting activities. Mot1 induces senescence in NIH 3T3 cells whereas Mot2 results in transformation. Mot2 has also been shown to increase proliferative life span and block induction

![Fig. 2. 2-D gel images showing selected up-regulated features (panel A), down-regulated features (panel B), and a feature with an altered migration (panel C).](image-url)
of senescence-associated-β-galactosidase activity in normal human diploid fibroblasts by interfering with p53 activity by blocking nuclear translocation of p53 and down-regulating p53-responsive genes (66, 67). Because up-regulation of HSP90 could account for down-regulation of HSP70 via HSF1, it suggests that it would be very important to determine what is the cause of the up-regulation of HSP90 and if it is because of stress of in vitro tissue culture, to determine the nature of this stress. It will also be interesting to determine whether HSP105/110 and 27 are also up-regulated by the same mechanism. One possible mechanism would be via a common transcription factor. It is also important to determine the exact form of HSP70 that we have found to be down-regulated and whether it corresponds to one of the mortalin proteins, particularly Mot2.

**Factors Affecting Protein Synthesis**—Two proteins, ERF1 (eukaryotic releasing factor 1, spot 677) and elongation factor-1-γ (spot 773), that may play a role in protein synthesis, were up-regulated upon senescence. ERF has been implicated in translation termination in eukaryotes (39). Although its function in translation termination remains obscure, it has been shown to promote a stop codon and ribosome-dependent hydrolysis of aminoacyl-tRNAs (40) and thus catalyzes the termination of protein synthesis at all three stop codons. It has been conserved remarkably during evolution suggesting that it may have an essential role in the termination of translation (41). Elongation factor-1-γ, a subunit of EF1, one of the G-proteins that mediate transport of aminoacyl-tRNA to the 80 S ribosomes during translation, has been found to be overexpressed in colorectal carcinomas and adenomas (42, 43). Therefore it was rather surprising that we found elongation factor-1-γ be up-regulated upon replicative senescence.

**Proteins Involved in Differentiation**—Two differentially regulated spots were identified as proteins belonging to the TUC (TOAD-64/Ulip/CRMP) family of intracellular phosphoproteins implicated in axon guidance and outgrowth and thereby regulation of neuronal differentiation (44–46). The TUC family consists of four 64-kDa isoforms known as TUC-1 (CRMP-1/Ulip-3), TUC-2 (CRMP-2/Ulip-2), TUC-3 (CRMP-3/Ulip-4), and TUC-4 (CRMP-4/Ulip-1). In addition, TUC-4β has been identified recently as a 75-kDa variant of TUC-4. The TUC proteins are up-regulated in the rat brain after embryonic day 12, a time that corresponds to the beginning of neurogenesis. TUC-4 is the most abundant member of this family and first detected in new post-mitotic neurons after neuronal birth (47), reaches peak expression levels during neurogenesis but is not expressed by neural progenitor cells. It is down-regulated in the adult brain but is re-expressed if axonal re-growth is triggered. It is also increased upon differentiation of PC12 cells by nerve growth factor and neuroblastoma cells by retinoic acid. In contrast to TUC-4, much less is known about the expression of the other TUC proteins. TUC-2 is expressed by both neurons and their progenitors. It is also slightly up-regulated upon treatment of PC12 cells with nerve growth factor. Recently TUC-2 was found to be associated with the microtubule bundles at the mitotic spindle and proposed to be involved in regulating microtubule dynamics (48). The TUC proteins are also required for the growth cone collapsing activity of semaphorin-3A, an extracellular guidance cue for axonal outgrowth, suggesting a role in the signal transduction pathway initiated by extracellular stimuli. Their importance in neuronal differentiation is further suggested by their homology to unc-33, a C. elegans gene that is required for normal axon outgrowth and guidance. They are also highly conserved across species; the rat TUC-2 protein has 98% identity with its chicken ortholog and 89% identity with its Xenopus laevis ortholog. The proteins identified by the proteomic analysis correspond to TUC-2 in spot 304 and TUC-4 in spot 495 that also contained the γ-subunit of the TCP-1 chaperone (45). TUC-2 was down-regulated 2.4-fold whereas TUC-4 was up-regulated when REFs underwent senescence, in accordance with the idea that TUC-4 is an early marker of post-mitotic neurons. Interestingly TUC-2 showed an opposite regulation to TUC-4; it was down-regulated in the post-mitotic senescent cells.

**Protein Turnover, Synthesis, and Modification**—Some differential spots were identified as subunits of the proteasome complex, the 26 S proteasome subunit p40.5 and RC10-II. The level of subunit p40.5 (spot 907) increases upon senescence whereas RC10-II both decreases in level (spot 1239) and shifts in its migration (spot 1348). The shift in migration is most likely to be because of changes in post-translational modification such as phosphorylation. The p40.5 subunit is expressed differentially between human luminal and myoepithelial cells (15). In contrast, changes in the migration pattern of the RC10-II have not been observed previously. It would therefore be very interesting to determine the site of phosphorylation, the kinase responsible, and how it is regulated.

Other proteins that could potentially be considered to be members of this family of differentially expressed proteins are O-GlcNAc transferase p110 subunit (spot 108), seryl-tRNA synthetase (spot 497), alanyl-tRNA synthetase (spot 102), and an ATP-dependent RNA helicase p47 (spot 677). All these proteins were identified as up-regulated proteins. O-GlcNAC transferase is capable of glycosylating both serine and threonine residues on nuclear and cytosolic proteins. On several proteins the O-GlcNAC and O-phosphorylation occur on the same or adjacent sites, and thus it is possible that one function of the addition of O-GlcNAC is to block phosphorylation at this site (49). Therefore the O-GlcNAC modification may have an important role in control of intracellular signaling. It is highly conserved, dynamic, and inducible. Disruption of O-GlcNAC transferase in mouse embryonic stem cells is lethal, demonstrating the importance of this modification.

**Protein Trafficking**—SEC23 identified in the down-regulated spot 153 in senescent cells has been proposed to be involved
in protein trafficking. It is a component of COPII, a cytosolic complex that is responsible for the formation of vesicles within the endoplasmic reticulum and involved in the anterograde transport of proteins to the Golgi (50, 51). In contrast to SEC23, transferrin (spot 290) and RA410, a vesicle transport-related protein (spot 426), both appear to be up-regulated upon senescence. None of these proteins have been found previously to be expressed differentially in senescence or tumorigenesis. Nevertheless, RA410 has been proposed to have a role in post-Golgi transport and participates in the ischemia-related stress response in astrocytes (52).

Metabolic Proteins—Two up-regulated spots (spots 122 and 127) that were identified to be the \( \alpha \)-subunit of the \( \alpha \)-glucosidase II complex were particularly interesting. \( \alpha \)-Glucosidase II is the soluble form of the enzyme and removes the \( \alpha \)-1,2-glucose and \( \alpha \)-1,3-glucose residues following transfer of \( \text{Glc}_3\text{Man}_9\text{GlcNAc}_2 \) to nascent polypeptides (53). The \( \alpha \)-glucosidases participate in glycoprotein folding mediated by calnexin and calreticulin by forming the monoglucosylated high mannose oligosaccharides required for interaction with chaperones. In addition to their role in N-glycan processing, these enzymes are involved intimately in quality control in the endoplasmic reticulum, a process that ensures proper folding of newly formed polypeptide chains leading to retention and/or degradation of incorrectly folded proteins (53). Interestingly it was shown recently that a 3' untranslated region of an \( \alpha \)-glucosidase-related mRNA was able to promote colony formation and immortalization in REFs and cooperate with an immortalization-defective mutant of SV40 T antigen to immortalize REFs (54). Moreover, a gene named klotho (55) that shares sequence homology to the \( \beta \)-glucosidase family of enzymes and encodes a secreted protein that appears to function outside cells has been identified recently. A defect in its expression in mice results in a syndrome that resembles human aging (short lifespan, infertility, arteriosclerosis, skin atrophy, osteoporosis, and emphysema) (55).

We also identified other differentially expressed metabolic proteins. Among these, 2-oxoglutarate dehydrogenase precursor (spot 104), 2-oxoglutarate dioxygenase \( \gamma \)-butyrobetaine (spot 493), and isopentenyl diphosphate dimethylallyl diphosphate isomerase (spot 1075) were up-regulated whereas GDP dissociation inhibitor of the Rho protein (spot 1054) was down-regulated. PRx IV (spot 1295), an enzyme with antioxidant function, was down-regulated whereas PRx III was found both to be down-regulated (spot 1239) and to undergo changes in mobility (spot 1351). Guanosine 5'-monophosphate synthetase (spot 332) was identified in a down-regulated spot.

### Validation of the 2-D Proteome Analysis

To validate the 2-D proteome analysis, REFs were passaged serially again using the strict 3T3 passaging regime to prepare extracts corresponding to P2, P3, P4, and P5 and analyzed for expression by 1-D Western blotting. To ensure that the extracts exhibited changes in expression of proteins known to be involved in replicative senescence, extracts were first analyzed for expression of cyclin A and p19\(^{ARF}\). As expected cyclin A was down-regulated, and p19\(^{ARF}\) was up-regulated as REFs underwent replicative senescence (Fig. 3A). Expression was then analyzed for some of the identified proteins for which antibodies were available.

Expression analysis of gelsolin, \( \alpha \)-glucosidase, and TUC-4 showed that they were up-regulated whereas TUC-2 was down-regulated in accordance with the proteome analysis (Fig. 3A). In contrast, even though HSP27 had been identified as an up-regulated feature, it was not found to be differential by 1-D Western blot analysis (see Fig. 2A and Fig. 3A). The most likely explanation for this is that HSP27 may have multiple isoforms and therefore be present as multiple spots on
the 2-D gels, and only one of these spots was differential. Thus the identity of the form of HSP27 that is expressed differentially upon replicative senescence remains to be determined. The 1-D Western blot experiments were repeated several times, and as expected each single experiment gave the same result. Further blotting experiments using specific antisera against the remainder of identified proteins are required to validate fully the differential proteome analysis.

We next undertook experiments to determine whether the changes at the protein level that were validated by 1-D Western blotting correlated with changes in mRNA by semiquantitative RT-PCR; the results are presented in Fig. 3B. Once again REFs were passaged serially using the strict 3T3 passaging regime and RNA extracted from cultures of P2, P3, P4, and P5 REFs. For these experiments p21WAF1/Sdi1/CIP1, known to be up-regulated upon replicative senescence (56), was used as a positive control. TUC-2 RNA decreased in accordance with the decrease in protein level. The level of TUC-4 and α-glucosidase RNA did not change whereas gelsolin RNA showed a slight increase. These results are consistent with the studies in S. cerevisiae and human liver, which have suggested that mRNA levels may correlate poorly with corresponding protein levels (13, 14).

Because TUC-4 and TUC-2 are two members of the same family of proteins but showed opposite regulation at the protein level, we analyzed expression of other members of this family. The results presented in Fig. 4A show that TUC-4β, a variant of TUC-4, was up-regulated, and TUC-1 was down-regulated, whereas TUC-3 was not present in the REFs. TUC-4β has been found previously to only be expressed in neural cells and cell lines of neural origin such as PC12, N1E-115, and neuro-2a cells. Interestingly both TUC-4 and TUC-4β were detected in all three of these cell lines, as well as in brain and REF extracts. The significance of the changes in expression of this family of proteins is not yet understood. Neither is it clear what is their function and whether they have overlapping functions. Nevertheless it is highly intriguing that the different family members show divergent but identical regulation in both neural differentiation and in fibroblasts upon replicative senescence.

We next examined whether some of the changes observed in replicative senescence also occur in oncogene-induced premature senescence, another commonly used system for studying senescence. In this model, REF52 cells, from an immortal rat cell line, are induced to senescence prematurely by ectopic expression of activated Ras. Cyclin A and p19ARF, known to be expressed differentially in both model systems, were used as a positive control. A direct correlation between the two models was found for α-glucosidase, gelsolin, and TUC-2. C, 20 μg of total cell lysates per sample were treated with Lambda protein phosphatase (BioLabs) prior to Western blotting.
A was down-regulated whereas p19ARF was up-regulated by 1-D Western blotting using antisera where available. We identify occasionally false positives, we validated this study turnover, and modification. Because proteomic studies do involved in trafficking, differentiation, and protein synthesis, heat shock, and metabolic proteins, as well as proteins in-...ion for 39 of these spots, comprising a variety of cytoskeletal, gelsolin and several times, and each experiment yielded the same result. Gelsolin and α-glucosidase were also found to be up-regulated when mouse embryo fibroblasts were passaged serially in vitro, and cultures ceased dividing and underwent replicative senescence (Fig. 5). Unfortunately these were the only two candidates for which the antibodies were capable of detecting the mouse protein. As more mouse specific antibodies become available it will be very interesting to validate the differential proteome analysis in the mouse system.

**SUMMARY**

Here we have presented a 2-D differential proteome analysis of replicative senescence in serially passaged rat embryo fibroblasts. Triplicate independent 2-D gels containing over 1200 spots each were run, curated, and analyzed. This revealed 49 spots, whose expression was altered more than 2-fold. Mass spectrometry yielded positive protein identification for 39 of these spots, comprising a variety of cytoskeletal, heat shock, and metabolic proteins, as well as proteins involved in trafficking, differentiation, and protein synthesis, turnover, and modification. Because proteomic studies do identify occasionally false positives, we validated this study by 1-D Western blotting using antisera where available. We next showed that some of the candidates were also expressed differentially in two other models of senescence, Ras-induced premature senescence of REF52 cells and replicative senescence of mouse embryo fibroblasts.

2-D gels have been used previously to compare the patterns of protein expression between normal and transformed cell lines. In studies where 400–1000 proteins were compared, it was reported that transformation caused a significant change in expression for 10–30% of the proteins (58, 59). In addition a number of marker proteins such as the tropomysin family of cytoskeletal proteins and the proliferation sensitive nuclear antigen for this process were identified (58, 60). These studies were extended by Garrels and Franza (58, 59) to generate a 2-D gel database for REF52 cells under a variety of growth states such as quiescence, rapidly dividing, and upon transformation by DNA and RNA tumor viruses. Moreover for a number of these 2-D spots, they determined the identity, the subcellular localization, and the post-translational modifications. Replicative senescence has not been analyzed previously by 2-D gels, and our study is one of the first such studies. In contrast to transformation, where 10–30% of the spots were differential, we found that only 49 spots of ~1200 were altered more than 2-fold (~4%). Because we have used triplicate curation to maximize reproducibility and eliminate false positives, it is possible that 4% differentials might be a slight underestimate for the proportion of differential spots, but it is considerably lower than the 10–30% differentials found in the REF52 studies. None of the differential spots that we have identified correspond to those identified previously by Garrels and Franza (58). This is perhaps not surprising, because REF52 cells are an immortal rat cell line, and the aim of our experiments was to identify changes that are the cause of the senescent phenotype, and these changes should not occur in REF52 cells when the immortal state is unaffected such as in quiescence, rapid cell division, and transformation.

Even though the proteins that we have identified have not been directly associated with replicative senescence previously, some of them, for example gelsolin, α-glucosidase, TUC, and heat shock proteins, have been linked to cell proliferation. Gelsolin has been proposed to be a tumor suppressor for breast cancer. A 3′-untranslated region of an α-glucosidase-related mRNA has been shown to be able to promote colony formation and immortalization in REFs and cooperate with an immortalization-defective mutant of SV40 T antigen to immortalize REFs (54). The TUC proteins are critical for neuronal differentiation, and it has been proposed by some that replicative senescence reflects a state of terminal differentiation (61) and that senescent fibroblasts are the equivalent of terminally differentiated end stage cells. However, the mechanisms by which these proteins modulate cell growth are unknown.

The finding that TUC genes are expressed differentially upon replicative senescence suggests another family of proteins that may be involved in this process. Treatment of neu-
robust cells with retinoic acid results in differential expression of the same TUC genes that we have observed. This treatment also affects expression of the p73 family of proteins, and p73 knock-out mice have a neuronal differentiation defect. p73 proteins are one of the two recently discovered members of the family of p53 related genes. We have already shown that p63 proteins, derived from the other member of this p53 family, are expressed differentially in replicative senescence and proposed that they may have a role in modulating p53 activity during replicative senescence (68). Moreover because the TUC-2 gene is down-regulated at the level of mRNA, it will be very interesting to determine whether this occurs at the level of transcription or stability and to determine the cause.

Therefore further experiments are necessary to determine whether any of the differentially expressed proteins, either singly or in combination, have a role in replicative senescence. The differentially expressed proteins also represent important starting points for determining the activities critical for the observed changes in expression whether they occur at the level of mRNA or protein or by post-translational modification and whether they are involved in replicative senescence of human cells. They may provide new markers for cancer diagnosis and therapeutics.

Acknowledgments—We are grateful to Alison Lloyd, Yasuo Ihara, and Helen Yin for the generous gift of antibodies. We are indebted to all our colleagues, Roy Kato in particular, for helpful discussions.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The on-line version of this article (available at http://www.mcponline.org) contains Supplemental Material.

To whom correspondence should be addressed. Tel.: 44-2078784101; Fax: 44-2078784040; E-mail: parrmjt@ludwig.ucl.ac.uk.

REFERENCES

1. Hanahan, D., and Weinberg, R. A. (2000) The hallmarks of cancer. Cell 100, 57–70
2. Hayflick, L. (1985) The cell biology of aging. Clin. Geriatr. Med. 1, 15–27
3. Counter, C. M., Hahn, W. C., Wei, W., Caddle, S. D., Beijsenbergen, R. L., Lansdorp, P. M., Sedivy, J. M., and Weinberg, R. A. (1998) Dissociation among in vitro telomerase activity, telomere maintenance, and cellular immortalization. Proc. Natl. Acad. Sci. U. S. A. 95, 14723–14728
4. Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijsenbergen, R. L., Brooks, M. W., and Weinberg, R. A. (1999) Creation of human tumour cells with defined genetic elements. Nature 400, 464–468
5. Kiyono, T., Foster, S. A., Koop, J. I., McDougall, J. K., Galloway, D. A., and Klingelhoitz, A. J. (1998) Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature 396, 84–88
6. O’Hare, M. J., Bond, J., Clarke, C., Takeuchi, Y., Atherton, A. J., Berry, C., Moody, J., Silver, A. R., Davies, D. C., Alsop, A. E., Neville, A. M., and Jat, P. S. (2001) Conditional immortalization of freshly isolated human mammary fibroblasts and endothelial cells. Proc. Natl. Acad. Sci. U. S. A. 98, 646–651
7. Jat, P. S., and Sharp, P. A. (1989) Cell lines established by a temperature-sensitive simian virus 40 large-T-antigen gene are growth restricted at the nonpermissive temperature. Mol. Cell. Biol. 9, 1672–1681
8. Gonos, E. S., Burns, J. S., Mazars, G. R., Kobama, A., Riley, T. E., Barnett, S. C., Zafarana, G., Ludwig, R. L., Ikrak, Z., Powell, A. J., and Jat, P. S. (1996) Rat embryo fibroblasts immortalized with simian virus 40 large T antigen undergo senescence upon its inactivation. Mol. Cell. Biol. 16, 5127–5138
9. Ikrak, Z., Norton, T., and Jat, P. S. (1994) The biological clock that measures the mitotic life-span of mouse embryo fibroblasts continues to function in the presence of simian virus 40 large tumor antigen. Proc. Natl. Acad. Sci. U. S. A. 91, 6448–6452
10. Cristofalo, V. J., Phillips, P. D., Sorger, T., and Gerhard, G. (1989) Alterations in the responsiveness of senescent cells to growth factors. J. Gerontol. 44, 55–62
11. Pereira-Smith, O. M., Robetorye, S., Ying, Y., and Orson, F. M. (1990) Hybrids from fusion of normal human T lymphocytes with immortal human cells exhibit limited life span. J. Cell. Physiol. 144, 546–549
12. Fodor, S. P., Rava, R. P., Huang, X. C., Pease, A. C., Holmes, C. P., and Adams, C. L. (1993) Multiplexed biochemical assays with biological chips. Nature 364, 555–556
13. Anderson, L., and Seilhamer, J. (1997) A comparison of selected mRNA and protein abundances in human liver. Electrophoresis 18, 533–537
14. Gygi, S. P., Rochon, Y., Franzia, B. R., and Aebersold, R. (1999) Correlation between protein and mRNA abundance in yeast. Mol. Cell. Biol. 19, 1720–1730
15. Page, M. J., Amess, B., Townsend, R. R., Parekh, R., Herath, A., Brusten, L., Zvelebil, M. J., Stein, R. C., Waterfield, M. D., Davies, S. C., and O’Hare, M. J. (1999) Proteomic definition of normal human luminal and myoepithelial breast cells purified from reduction mammaplasties. Proc. Natl. Acad. Sci. U. S. A. 96, 12589–12594
16. Wilm, M., Shevchenko, A., Houthaeve, T., Schweger, L., Fotsis, T., and Mann, M. (1996) Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. Nature 379, 466–469
17. Todaro, G. J., and Green, H. (1963) Quantitative studies of the growth of mouse embryo cells in culture and their development into established cell lines. J. Cell Biol. 17, 299–313
18. Harris, R. A., Yang, A., Stein, R. C., Lucy, K., Brusten, L., Herath, A., Parekh, R., Waterfield, M. D., O’Hare, M. J., Neville, M. A., Page, M. J., and Zvelebil, M. J. (2002) Cluster analysis of an extensive human breast cancer cell line protein expression map database. Proteomics 2, 212–223
19. Kwiatkowski, D. J. (1999) Functions of gelsolin: motility, signaling, apoptosis, cancer. Curr. Opin. Cell. Biol. 11, 103–108
20. Arora, P. D., Janney, P. A., and McCulloch, C. A. (1999) A role for gelsolin in stress fiber-dependent cell contraction. Exp. Cell Res. 250, 155–167
21. Kothakota, S., Azuma, T., Reinhard, C., Klippe1, A., Tang, J., Chu, K., McCurry, T. J., Kirschner, M. W., Koths, K., Kwiatkowski, D. J., and Williams, L. T. (1997) Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science 276, 294–298
22. Kusano, H., Shimizu, S., Koya, R. C., Fujita, H., Kamada, S., Kuzumaki, N., and Tsujimoto, Y. (2000) Human gelsolin prevents apoptosis by inhibiting apoptotic mitochondrial changes via closing VDAC. Oncogene 19, 4807–4814
23. Vandelckchove, J., Bauw, G., Vancompernolle, K., Honore, B., and Celis, J. (1990) Comparative two-dimensional gel analysis and microsequencing identifies gelsolin as one of the most prominent down-regulated markers of transformed human fibroblast and epithelial cells. J. Cell Biol. 111, 95–102
24. Lee, H. K., Driscoll, D., Asch, H., Asch, B., and Zhang, P. J. (1999) Downregulated gelsolin expression in hyperplastic and neoplastic lesions of the prostate. Prostate 40, 14–19
25. Dong, Y., Asch, H. L., Medina, D., Ip, C., Ip, M., Guzman, R., and Asch, B. B. (1998) Concurrent deregulation of gelsolin and cyclin D1 in the majority of human and rodent breast cancers. Int. J. Cancer 81, 930–938
26. Asch, H. L., Head, K., Dong, Y., Notoli, F., Winston, J. S., Connolly, J. L., and Asch, B. B. (1996) Widespread loss of gelsolin in breast cancers of humans, mice, and rats. Cancer Res. 56, 4841–4845
27. Kannan, K., Kaminshi, N., Rechavi, G., Jakob-Hirsch, J., Amarigli, N., and Givol, D. (2001) DNA microarray analysis of genes involved in p53 me-
Proteomic Analysis of Replicative Senescence

diated apoptosis: activation of Apaf-1. Oncogene 20, 3449–3455
28. Tsukita, S., and Yokemura, S. (1999) Cortical actin organization: lessons from ERM (ezrin/radixin/moesin) proteins. J. Biol. Chem. 274, 34507–34510
29. Mullins, R. D. (2000) How WASP-family proteins and the Arp2/3 complex convert intracellular signals into cytoskeletal structures. Curr. Opin. Cell Biol. 12, 91–96
30. Mullins, R. D., and Pollard, T. D. (1999) Structure and function of the Arp2/3 complex. Curr. Opin. Struct. Biol. 9, 244–249
31. Welch, M. D. (1999) The world according to Arp: regulation of actin nucleation by the Arp2/3 complex. Trends Cell Biol. 9:423–427
32. Lindquist, S. (1986) The heat-shock response. Annu. Rev. Biochem. 55, 1151–1191
33. Hendrick, J. P., and Hartf, F. U. (1993) Molecular chaperone functions of heat-shock proteins. Annu. Rev. Biochem. 62, 349–384
34. Verbeke, P., Fonager, J., Clark, B. F., and Rattan, S. I. (2001) Heat shock response and ageing: mechanisms and applications. Cell Biol. Int. 25, 845–857
35. Rattner, J. B. (1991) hsp70 is localized to the centrosome of dividing HeLa cells. Exp. Cell Res. 195, 110–113
36. Hatayama, T., Honda, K., and Yukkoka, M. (1986) HeLa cells synthesize a specific heat shock protein upon exposure to heat shock at 42 °C but not at 45 °C. Biochem. Biophys. Res. Commun. 137, 957–963
37. Imagawa, M., Tsuchiyaa, T., and Nishihara, T. (1999) Identification of inducible genes at the early stage of adipocyte differentiation of 3T3-L1 cells. Biochem. Biophys. Res. Commun. 254, 1476–1483
38. Piotrowicz, R. S., Weber, L. A., Hickey, E., and Levin, E. G. (1995) Accelerated growth and senescence of arterial endothelial cells expressing the small molecular weight heat-shock protein HSP27. Faseb J. 9, 1079–1084
39. Frolova, L., Le Goff, X., Rasmussen, H. H., Cherepin, S., Druegon, G., Kress, M., Arman, I., Haenendi, A. C., Celes, J. E., Philippo, M., Justesen, J., and Kisselev, L. (1994) A highly conserved eukaryotic protein family possessing properties of polypeptide chain release factor. Nature 372, 701–703
40. Frolova, L. Y., Tsivkovskiy, R. Y., Sivolobova, G. F., Oparina, N. Y., Serpinsky, O. I., Blinov, M. V., Tatkovo, S. I., and Kisselev, L. L. (1999) Mutations in the highly conserved GGG motif of class 1 polypeptide release factors abolish ability of human eRF1 to trigger peptidyl-tRNA hydrolysis. RNA 5, 1014–1020
41. Guenet, L., Toutain, B., Guillieret, I., Chauvel, B., Deaven, L. L., Longmire, J. L., Le Gali, J. Y., David, V., and Le Treut, A. (1999) Human release factor eRF1: structural organization of the unique functional gene on chromosome 5 and of the three processed pseudogenes. FEBS Lett. 454, 131–136
42. Mimori, K., Mori, M., Inoue, H., Ueo, H., Mafune, K., Akiyoshi, T., and Sugimachi, K. (1998) Elongation factor 1 gamma mRNA expression in esophageal carcinoma. Gut 38, 66–70
43. Mimori, K., Mori, M., Tanaka, S., Akiyoshi, T., and Sugimachi, K. (1995) The overexpression of elongation factor 1 gamma mRNA in gastric cancer. Cancer 75, 1446–1449
44. Byk, T., Ozon, S., and Sobel A. (1998) The Ulp1 family phosphoproteins—common and specific properties. Eur. J. Biochem. 254, 14–24
45. Wang, L. H., and Strittmatter, S. M. (1996) A family of rat CRM1 genes is differentially expressed in the nervous system. J. Neurosci. 16, 6197–6207
46. Quinn, C. C., Gray, G. E., and Hockfield, S. (1999) A family of proteins implicated in axon guidance and outgrowth. J. Neurobiol. 41, 158–164
47. Minturn, J. E., Fryer, H. J., Geschwind, D. H., and Hockfield, S. (1999) TOAD-64, a gene expressed early in neuronal differentiation in the rat, is related to unc-33, A. elegans gene involved in axon outgrowth. J. Neurosci. 15, 6757–6766
48. Gu, Y., and Ihara, Y. (2000) Evidence that collapsin response mediator protein-2 is involved in the dynamics of microtubules. J. Biol. Chem. 275, 17917–17920
49. Wells, L., Vosseller, K., and Hart, G. W. (2001) Glycosylation of nucleocytoplasmic proteins: signal transduction and O-GlcNAc. Science 291, 2376–2378
50. Barlowe, C. (1995) COPII: a membrane coat that forms endoplasmic reticulum–derived vesicles. FEBS Lett. 369, 93–96
51. Nickell, W., Brugger, B., and Wieland, F. T. (1998) Protein and lipid sorting between the endoplasmic reticulum and the Golgi complex. Semin. Cell Dev. Biol. 9, 493–501
52. Matsu, N., Ogawa, S., Takagi, T., Watana, A., Mori, T., Matsuayma, T., Pinsky, D. J., Stern, D. M., and Tohymaya, M. (1997) Cloning of a putative vesicle transport-related protein, RA410, from cultured rat astrocytes and its expression in ischemic rat brain. J. Biol. Chem. 272, 16438–16444
53. Herscovichs, A. (1999) Importance of glycosidases in mammalian glycoprotein biosynthesis. Biochim. Biophys. Acta 1473, 96–107
54. Powell, A. J., Daron, A. J., Gonos, E. S., Lam, E. W., Peden, K. W., and Jat, P. S. (1999) Different functions are required for initiation and maintenance of immortalization of rat embryo fibroblasts by SV40 large T antigen. Oncogene 18, 7343–7350
55. Kuro-o, M., Matsumura, Y., Aiawa, H., Kawaguchi, H., Suga, T., Utsugi, T., Ohyama, Y., Kurabayashi, M., Kaname, T., Kume, E., Iwasaki, H., Iida, A., Shiraki-Iida, T., Nishikawa, S., Nagai, R., and Nabhshima, Y. I. (1997) Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature 390, 45–51
56. Bringold, F., and Serrano, M. (2000) Tumor suppressors and oncogenes in cellular senescence. Exp. Gerontol. 35, 317–329
57. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997) Oncogenic Ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88, 593–602
58. Garrels, J. I., and Franca, B. R., Jr. (1989) The REF52 protein database. Methods of database construction and analysis using the QUEST system and characterizations of protein patterns from proliferating and quiescent REF52 cells. J. Biol. Chem. 264, 5283–5298
59. Garrels, J. I., and Franca, B. R., Jr. (1989) Transformation-sensitive and growth-related changes of protein synthesis in REF52 cells. A two-dimensional gel analysis of SV40, adenovirus–, and Kirsten murine sarcoma virus-transformed rat cells using the REF52 protein database. J. Biol. Chem. 264, 5299–5312
60. Morris, G. F., and Mathews, M. B. (1989) Regulation of proliferating cell nuclear antigen during the cell cycle. J. Biol. Chem. 264, 13856–13864
61. Bayreuther, K., Rodemann, H. P., Hommel, R., Dittmann, K., Albiez, M., and Franz, P. I. (1998) Human skin fibroblasts in vitro differentiate along a terminal cell lineage. Proc. Natl. Acad. Sci. U. S. A. 95, 5112–5116
62. Wright, W. E., and Shay, J. W. (2000) Telomere dynamics in cancer progression and prevention: fundamental differences in human and mouse telomere biology. Nat. Med. 6, 849–851
63. Serrano, M., and Biscas, M. A. (2001) Putting the stress on senescence. Nat. Rev. Mol. Cell Biol. 2, 981–989