Modification Form for Permit BIO-UWO-0147

Permit Holder: Sung Kim

Approved Personnel
(Please stroke out any personnel to be removed)

Boram Ham

Anthony Bruni

Sarah Spanton

Andrew Martins

Soon-Duck Ha

Additional Personnel
(Please list additional personnel here)

Sangwook Park (spark36@uwo.ca)

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. *
Approved Microorganisms	P. aeurogenosa, S. typhimurium, actobacillus rhamnosus, E. coli EC1000, E.Coli BL21.	
Approved Cells	Human (primary), rodent (primary), human (established), THP-1, rodent (established), ~~RAW 2649~~ RAW 264.7, HEK293, HepG2, CHO/CHO-K1, L929, Caco2, u937, NPC-1 deficient fibroblast (GMO3123) and wildtype	KBM-7
Approved Use of Human Source Material	Blood (whole), PARF-CFP, mRFP-Rab7, mRFP-Rab5	
Approved GMO	pTRK830, pOR128, pTRK669	pDisrup 8.0-lox (Retrovirus Vector) pBABE-cre (" ")
Approved use of Animals	mice	
Approved Toxin(s)	cholera, diphtheria, CONT'D, Anthratoxin	

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED.*

As the principal investigator, I have ensured that all of the personnel named on the form have been trained. I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca.

Signature of Permit Holder: 

Classification: 2

Date of Last Biohazardous Agents Registry Form: Apr 28, 2008

Date of Last Modification (if applicable): Nov 23, 2009

BioSafety Officer(s): _____

Chair, Biohazards Subcommittee: _____

We will trap genes using our pDisrup-8.0-lox retrovirus vector as we published in BioTechniques (see attached article published in 2007) using the cell line KBM7.

We will randomly knockdown genes in haploid KBM7 cells using the gene trapping retroviral vector pDisrup8.0-lox. We will then screen for their phenotypes in cell death susceptibility to anthrax lethal toxin. Genes trapped by the retroviral vector will be identified in clones resistant to cell death to find novel genes involved in cell death.

KBM7 cells were derived from the bone marrow of a patient with myeloid leukemia (Experimental Cell Res 252, 273, 1999). The retrovial vector was constructed from a retrovirus vector obtained from Dr. G.P. Nolan as indicated the BioTechnique paper attached.

Mutagenesis by retroviral insertion in chemical mutagen-generated quasi-haploid mammalian cells

Sung O. Kim[1], Soon-Duck Ha[1], Sangun Lee[2], Sarah Stanton[2], Bruce Beutler[2], and Jiahuai Han[2]

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Diploidy is a major obstacle to the mutagenic analysis of function in cultured mammalian cells. Here, we show that 6–8 rounds of chemical mutagenesis generates quasi-haploid cells that can be used as targets for insertional mutagenesis using a specially designed retroviral vector that permits rapid identification of disrupted genes in each cell that bears a phenotype of interest. The utility of combined chemical and insertional mutagenesis is illustrated by the identification of novel host genes that are required for macrophage sensitivity to anthrax lethal factor.

INTRODUCTION

The creation of novel phenotypes and identification of the mutations responsible has led to some of the most important advances in biology. In mammalian systems, phenotypes may be created either by modifying the germline or by modifying cultured cells, using either chemical or insertional mutagens. Germline mutagenesis can reveal phenotypes that are impossible to produce in culture (e.g., behavioral phenotypes or phenotypes related to development). However, many basic biological phenomena such as cell cycle regulation and cell death can be better explored using cultured cells. Germline mutagenesis is limited by the very low rate at which mutations can be produced (by present estimates, perhaps 100 times the background rate). In vitro mutagenesis in cultured cells can be expected to yield far higher rates of mutation than the germline approach. Because of the biological complexity of animal models and the time and expense involved in breeding, cells and cell lines are often preferred when cellular questions are at issue. Unfortunately, forward genetic studies in cultured mammalian cells, as presently practiced, have serious limitations (1–10).

Given that the cells used are diploid, only a single copy of each autosomal gene is generally destroyed by mutagenesis in vitro, and breeding cannot be used to achieve homozygosity for mutations as it can be in germline mutagenesis. In many instances, a dominant phenotype is not rendered and, indeed, cannot be rendered by mutation. This problem has led to develop antisense library approaches (5,9,11,12), and rescue methods have been developed to quickly identify which antisense cDNA is responsible for a given phenotype (9). But for many genes, antisense RNA expression is not effective in blocking expression. The same problem is present if a modified method, random homozygous knockout (RHKO), is used to produce a phenotype (8). In recent years, the use of small interfering RNA (siRNA) library has attracted a great deal of attention as a powerful tool for functional identification of genes (13–15). siRNA is clearly much more efficient than antisense RNA in knocking down gene expression; however, there are still some drawbacks. One study has shown that sequence identity of as few as 11 to 12 nucleotides between an interfering RNA and a messenger RNA (mRNA) may be sufficient for interference to occur (16). If this is true, then cross-reactivity, which is referred to as the off-target effect, could be a substantial problem (17). Although RNA interference (RNAi) targeting efficiency is much better than antisense, it is clear—as in the case of worms or flies—that different genes in mammalian cells are turned down with differing efficiencies. The potential interferon response to siRNA expression in mammalian cells could also interfere with some genetic screens (18–20). Recently, two groups of investigators generated embryonic stem (ES) cell libraries with genome-wide biallelic mutations (21,22). The increase in the rate of loss-of-heterozygosity (LOH) in Bloom's syndrome gene (*Blm*) deficient cells was used in their strategy to generate these biallelic mutations. *Blm*-deficient cells carrying heterozygous mutations segregate into homozygous daughters in vitro and in vivo, presumably through mitotic recombination between nonsister chromatids. One group used *Blm* knockout ES cells and another group developed a tetracycline-inducible system to transiently knockout *Blm*

[1]University of Western Ontario, London, ON, Canada and [2]The Scripps Research Institute, La Jolla, CA, USA

in ES cells. The rate of LOH in *Blm*-deficient ES cells is 4.2×10^{-4} and 2.3×10^{-4} determined by the two groups, respectively, which is approximately a 20-fold increase from the wild-type ES cells. In order to allow the LOH to occur in most of the loci, both groups passed the mutated cells a number of generations. As a result, the libraries contained approximately 5×10^8 cells, and among them, 0.01%–0.1% cells containing biallelic mutations. Although the rates of LOH were quite low in these studies, considering the hypomorphic allele used by the first group and possible leaky inducible system used by the second group, the rate of mitotic recombination could have been higher, and therefore, using LOH to generate biallelic mutations can be a very hopeful approach. However, how the method will practically be applied to other cell types remains to be examined.

Retroviral insertion can create a single mutation in a cell, and the inserted retrovirus offers a tag with which to find the mutated gene with relative ease (23,24). In principle, insertional mutagenesis would be very effective if haploid mammalian cells could be created in culture. High rates of mutations can be most efficiently achieved by chemical mutagens because of their potency and because they can be applied to culture cells multiple times.

Here, we describe a method that utilizes the power of chemical mutagenesis in vitro to generate a pool of cells that can be regarded as quasi-haploid. We then use a specifically designed retroviral vector to randomly disrupt genes that have been rendered haploid, permitting rapid identification of each mutation that causes a phenotype of interest.

MATERIALS AND METHODS

Mutagensis with Chemical Mutagens

N-ethyl-N-nitrosourea (ENU; Sigma, St. Louis, MO, USA) was dissolved in equal portions of 95% ethanol and PCS buffer (50 mM sodium citrate, 100 mM sodium phosphate, pH 5.0). O^6-benzylguanine (O^6-BG) was dissolved in dimethyl sulfoxide (DMSO). Approximately 50% confluence cells in a 10-mm dish were pretreated with 10 µM O^6-BG for 12 h and then treated with 0.35 mg/mL ENU for 2 h in the presence of O^6-BG. The cells were washed and incubated with fresh medium containing O^6-BG for 24 h to recovery. The cells were plated onto a 10-mm dish at approximately 1000 cells/dish. Four dishes were used to measure mutation frequency, and the other two were used for the next round of mutagenesis. To detect hypoxanthine phosphoribosyl transferase (*Hprt*) null mutants, individual clones were picked from the 100-mm dish into 96-well plates. After culturing these clones for 24 h, 10 µM 6-thioguanine (6-TG) were added to the medium, and 6-TG-resistant clones were scored 1 week later. Mutagenesis with 1.5 µM acridine mutagen ICR-191 was performed the same as ENU, except the time of treatment was 24 h.

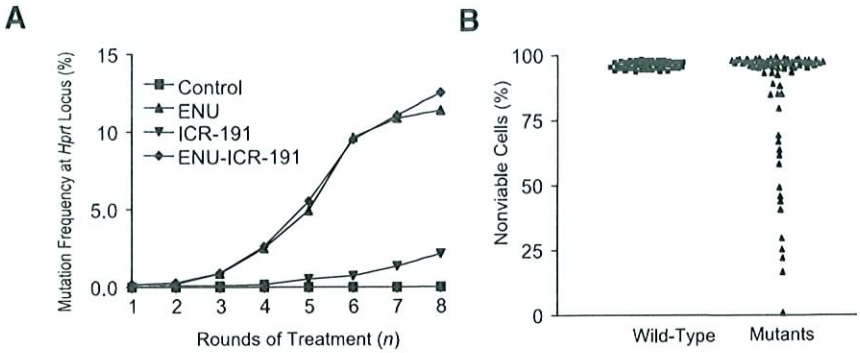

Figure 1. Determination of chemical mutagenesis frequency and selection of lethal toxin-sensitive clones. (A) Mutation frequency in RAW 264.7 cells treated with N-ethyl-N-nitrosourea (ENU) or acridine mutagen ICR-191 or ENU plus ICR. Cells were treated with different rounds of chemical mutagens. The mutation frequency was determined by the percentage of cells that had a recessive mutation on the hypoxanthine phosphoribosyl transferase (*Hprt*) gene. (B) The sensitivity to lethal toxin-induced cell death of individual clones treated with or without chemical mutagens. The viabilities of each of individual clones of 50 parental and 72 chemically mutated clones treated with 500 ng/mL lethal factor (LF) and protective antigen (PA) were measured by crystal violet uptake.

Mutagenesis with Retrovirus

The pDisrup 8 retroviral vector was constructed as described previously, except a ribozyme sequence was incorporated into the vector (25). A blasticidin-resistant gene was used in this vector because blasticidin selection requires much less time than G418 selection. The virus preparation and infection were performed as described (25). Briefly, pDisrup 8 recombinant retroviruses were generated in Phoenix amphotropic producer cells using the calcium phosphate method of transfection. Viruses were produced at 32°C, and virus-containing medium was collected 24 h posttransfection and filtered through a 0.45-µm filter. The haploid RAW 264.7 cell pool was plated in 6-well plates at a density of 5×10^5 cells/well. Retroviral infection was performed by replacing medium with 2 mL pDisrup 8 virus (containing 4 µg Polybrene®/mL; Sigma), followed by centrifugation of the 6-well plates at 859× *g* for 30 min at 32°C. The infection efficiency was estimated by paralleled experiments using a similar retrovirus expressing green fluorescent protein. Blasticidin (10 µg/mL) was used to select blasticidin-resistant cells.

3′ Rapid Amplification of cDNA Ends

The portion of the endogenous gene that was fused with the *blasticidin+* gene was amplified by the 3′ rapid amplification of cDNA ends (3′-RACE) technique. Total RNA was isolated, and reverse transcription was performed with the primer 5′-CCAGTGAGCAGAGTGACGAG GACTCGAGCTCAAGC$[T]_{17}$-3′. A nested PCR was performed with the resulting reverse transcription product with the following primers: P1/Q1 (5′-AAAGCGATAGTGAAGGACAGT

GA-3′ and 5′-CCAGTGAGCAGAGT GACG-3′) and P2/Q2 (5′-TGCT GCCCTCTGGTTATGTGTGG-3′ and 5′-GAGGACTCG AGCTCAAGC-3′). P1 and P2 are located on the *blasticidin+* gene, while Q1 and Q2 are on the anchor sequence of the reverse transcription, QT. The PCR products of 3′-RACE and reverse transcription PCR (RT-PCR) were directly sequenced.

Small Interfering RNA

siRNAs targeting cytohesin-4 or J κ recombination signal binding protein (Rbp-jκ), were stably expressed in RAW 264.7 cells by using pSuper vector (OligoEngine, Seattle, WA, USA) encoding a neomycin-resistant gene. All neomycin-resistant clones were pooled and analyzed for anthrax lethal toxin (LeTx) sensitivity. The sequence of the siRNAs are 5′-AAGGAGCTACAGCAGATCAAA -3′ for cytohesin-4 and 5′-AAGGAGAGGAGTTCACAGTTA-3′ for Rbp-jκ. Predesigned siRNAs were purchased from Ambion (Austin, TX, USA) for Atp6v0c and Laptm5 and Dharmacon (Chicago, IL, USA) for hypoxia-inducible factor 1-α subunit (Hif1-α) and were transiently transfected in RAW 264.7 cells by using siPORT™ Amine transfection reagent (Ambion). LeTx sensitivity was analyzed 48 h after the transfection.

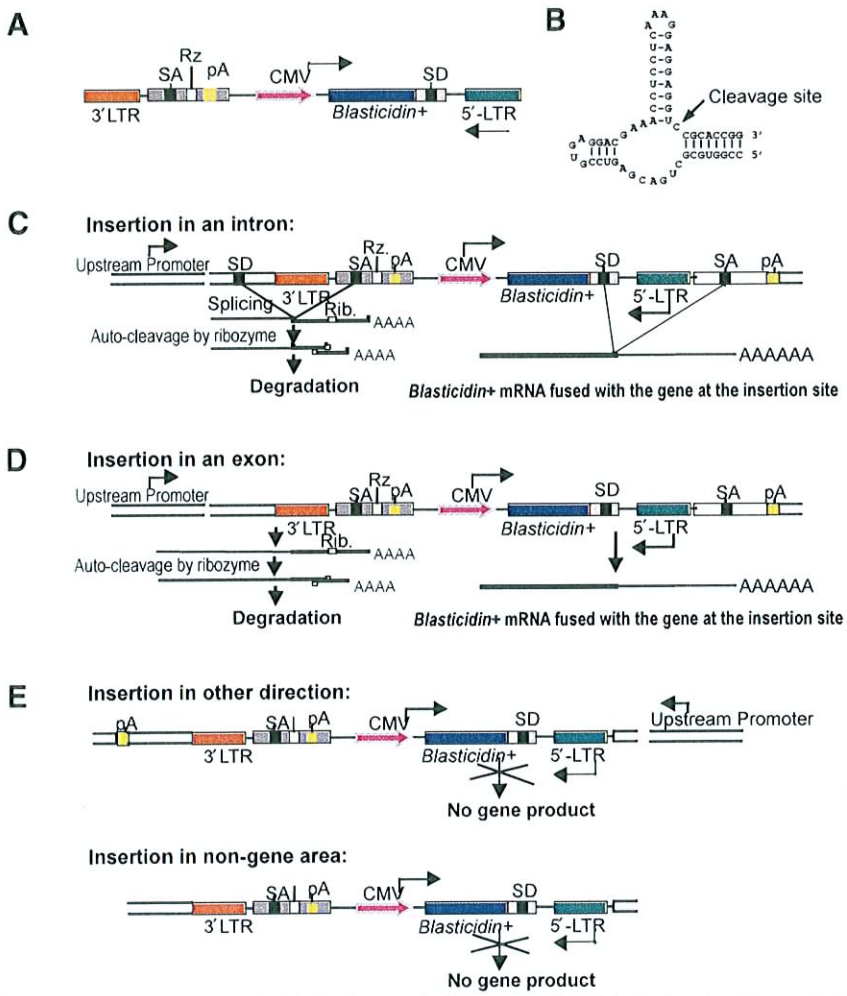

Figure 2. Representation of pDisrup 8 retroviral vector-mediated insertional mutagenesis. (A) Diagram of the retroviral vector pDisrup 8 used for gene knockout. Rz, ribozyme sequence; SD, splicing donor; SA, splicing acceptor; pA, poly(A) signal sequence; CMV, cytomegalovirus promoter; *blasticidin+*, blasticidin-resistant gene; (B) Sequence of the ribozyme in the vector. (C) Viral insertion in an intron. Opened bars are flanking sequence of genome at the insertion site. The upstream SD of the endogenous gene splices with a vector-encoded SA and the transcript ends at the vector pA. The Rz located 3′ to the SA sequence cleaves the transcript and results in no gene product. The SD downstream of the *blasticidin+* coding sequence splices with endogenous SA to generate fused messenger RNA (mRNA) capable of expressing full-length *blasticidin+* gene product. (D) Viral insertion in an exon. The transcript initiated by the endogenous gene directly fuse with the viral gene to end at the vector pA. The ribozyme cleaves this transcript, and no gene product will be generated. The *blasticidin+* transcript will join exons downstream of the insertion site by fusion or splicing events, depending on the presence of a downstream SA. Full-length *blasticidin+* gene product is produced. (E) Insertion occurs in the other direction (either intron or exon) or not in a gene. The CMV promoter driven transcript of *blasticidin+* gene lacks a pA, which results in degradation of the transcript. No *blasticidin+* gene product is produced. LTR, long terminal repeat.

Cell Culture and Viability Assay

A single clone of RAW 264.7 cells was isolated and used as parental cells for the mutagenesis. The mutated cells generated at every experimental step were frozen to store at a very early passage to maintain original mutants in the event that spontaneous reversions occurred during cell culture. LeTx-induced cell death was measured using crystal violet uptake as described (26). Components of lethal toxin, lethal factor (LF), and protective antigen (PA) were obtained from List Biological Laboratories (Campbell, CA, USA) and used at 1 μg/mL unless otherwise indicated.

RESULTS

Development of Quasi-Haploid Cells Through High-Intensity Chemical Mutagenesis

A quasi-haploid culture can be created by generating loss-of-function mutations that affect one allele of most or all genes within a culture of limited complexity. The number of cells that are needed to create such pool depends upon the percentage of monoallelic mutations introduced within each cell. If 10% of genes are mutated on one allele in each cell, and if mutations occur at random, a pool of 40 cells should contain monoallelic mutations of 98.5% of all genes [$1-(1-X)^n$; X, mutation rate in each cell; n, number of cells]. Thus, it is feasible to obtain a pool of cells with a quasi-haploid background if we can create functionally null monoallelic

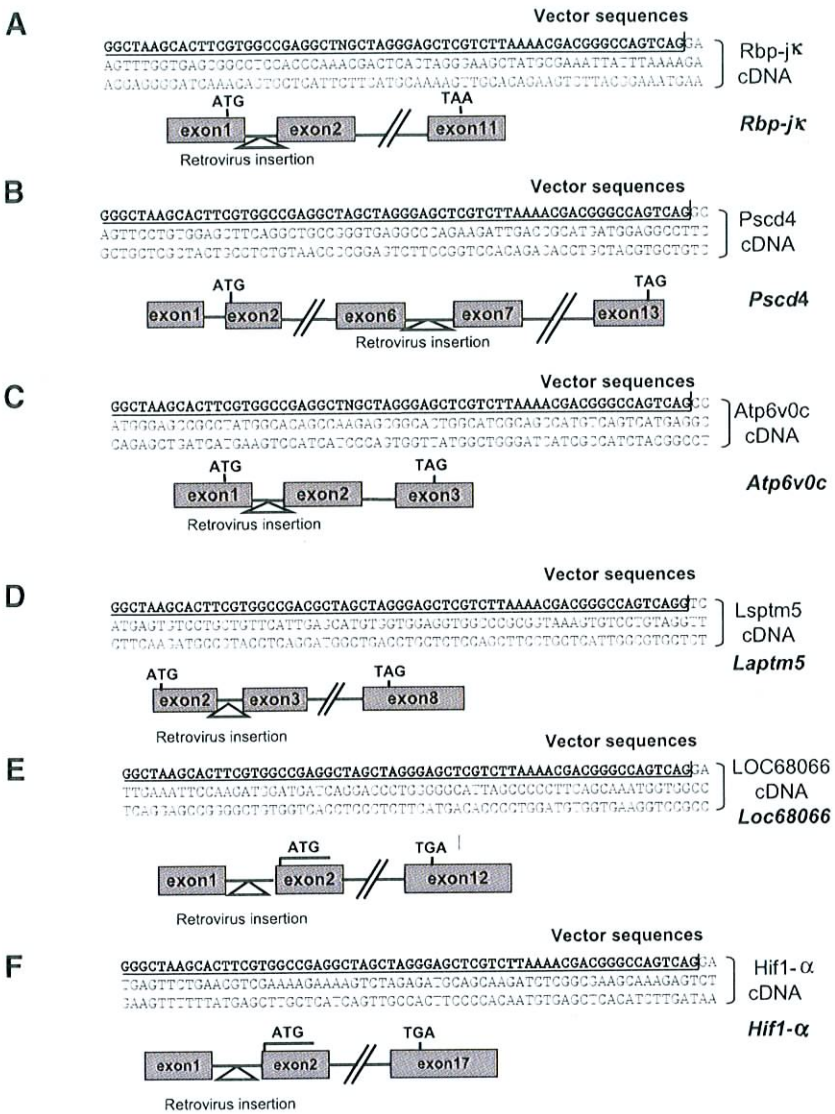

Figure 3. The fused messenger RNA (mRNA) of *blasticidin*+ and an endogenous gene in six anthrax lethal toxin (LeTx)-resistance RAW 264.7 clones were amplified by 3′ rapid amplification of cDNA ends (3′-RACE) and analyzed by DNA sequencing. (A) *Blasticidin*+ encoding by retroviral vector was fused with J κ recombination signal binding protein (Rbp-jκ) mRNA. The insertion occurred in an intron between exon 1 and exon 2, which results in a disruption of one allele of *Rbp-jκ*. (B) *Blasticidin*+ mRNA was fused with cytohesin-4 (Pscd4) mRNA. The viral insertion occurred between exon 6 and exon 7. One allele of *Pscd4* is disrupted. (C) *Balsticidin*+ mRNA was fused with subunit c of vacuolar H^+-ATPases (Atp6v0c) mRNA. The insertion is between exon 1 and exon 2. One allele of *Atp6v0c* was disrupted by retrovirus. (D) *Balsticidin*+ mRNA was fused with lysosomal-associated protein transmembrane 5 (Laptm5) mRNA. The insertion is between exon 2 and exon 3. (E) *Balsticidin*+ mRNA was fused with a hypothetical protein homolog to human mitochondrial carrier CGI-69 (LOC68066) mRNA. The insertion is between exon 1 and exon 2. (F) *Balsticidin*+ mRNA was fused with hypoxia-inducible factor 1-α subunit (Hif1-α) mRNA. The insertion is between exon 1 and exon 2.

mutations in 10% of genes in each cell of a relatively small population. However, it is not intuitively clear that such a high rate of mutation can be achieved.

To examine whether multiple round of mutagenesis can create cells with single allele mutations affecting 10% of all genes, we used a male mouse-derived monocytic cell line RAW 264.7 cells. The karyotype of RAW 264.7 cells was verified to be diploid in nature based on chromosome-spreading and counting analysis (data not shown). RAW 264.7 cells were exposed to several rounds of 0.35 mg/mL ENU or 1.5 μM ICR-191 or both ENU and ICR-191. O^6-BG (10 μM) pretreatment was included to increase the mutation frequency of ENU (27). We tested for loss-of-function mutations at the hemizygous (X-linked) *Hprt* locus by growing cells in 10 μM 6-TG containing medium after each round of mutagenesis.

The deleterious mutation frequency at the *Hprt* locus reached 10^{-1} after 6–8 rounds of exposure to ENU (Figure 1A). ICR-191-induced loss-of-function mutations of *Hprt* were, as previously reported (28), less frequent than loss-of-function mutations induced by ENU. The combination of the two mutagens produced no significant additive effect in RAW 264.7 cells (Figure 1A). Notably, the mutagenic efficiency was increased with repeated cycles of mutagenesis, which was peaked at the fifth cycle. This phenomenon may reflect progressive mutational degradation of repair systems (e.g., defects in DNA mismatch repair proteins) that oppose mutational pressure. The asymptotic decline in mutagenic efficiency observed after the fifth cycle presumably reflects a limitation in the amount of mutations that the cell can tolerate since we observed less cell recovery in the later cycles.

Approximately 1 month was required to complete eight rounds of mutagenesis. While some parts of the genome may be more resistant to chemical mutagenesis than others and functional heterozygosity is known to be forbidden at some loci (29) and while compound homozygosity for null mutations may have also been achieved at some loci (10,30), these should only take a very small percentage of whole genome. Therefore, based on the loss-of-function mutations of *Hprt*, we consider that approximately 12% of the genome was rendered functionally haploid by repeated cycles of exposure to ENU (Figure 1A). However, the tolerance can be attributed to loss- or gain-of-function of genes other than *Hprt*. In addition, a month of continuous culture with repeated ENU treatments can provide ample opportunity for genetic drift, a fortuitous progressive overgrowth of a few clones marked by the resistance mutation. In this case, the saturated ENU mutation rate will be lower than we expected, and larger numbers of

cells are required to be pooled to reach a quasi-haploid background.

Selecting the Mutated Cells for the Pool of Quasi-Haploid Cells

The quasi-haploid background needs to be created as the starting point for insertional mutagenesis, in the expectation that most genes will be susceptible to functional knockout only by a single insertional event. In order to achieve this, the cells that constitute the pool of quasi-haploid cells should be a set of phenotypically normal clones.

In the present example, the phenotype to be identified is sensitivity to LeTx-induced cell death. Accordingly, a polyclonal population of cells with residual sensitivity to lethal toxin-induced death needs to be culled from the starting population. Seventy-two individual clones were isolated at random from the pool of cells that survived eight rounds of ENU and ICR-191 treatment. Each of these clones was tested for susceptibility to lethal toxin-induced cell death in comparison with parental wild-type RAW 264.7 cells. About 50 clones showed sensitivity to lethal toxin equal to that of the wild-type RAW 264.7 cells. The remaining clones had some alteration in their sensitivity to lethal toxin-induced cell death (Figure 1B). Forty-two lethal toxin-sensitive clones were then mixed to yield the quasi-haploid cell population that was used as a target for the following insertional mutagenesis.

Phenotype-Based Screen of Retroviral Insertion-Induced Mutations in Quasi-Haploid Cells

Retroviral insertion can abolish gene expression or produce a truncated gene product depending upon the site of insertion. Truncation of the gene product might yield unpredictable results, since some truncated proteins might display dominant properties, causing either a gain- or loss-of-function. In order to produce gene deletion and avoid gene truncation, we incorporated a self-cleavage ribozyme sequence into the retroviral vector pDisrup 8 (Figure 2A). The sequence of the self-cleavage ribozyme in the vector was shown in Figure 2B. Any transcript containing this ribozyme will be destroyed, because the mRNA will be self-cleaved and subsequently degraded. Previous studies have shown that this type of self-cleaving ribozyme results in a complete cleavage of RNA (31,32), and in our experience, all of the transcript incorporating the ribozyme encoding sequence, generated by a cytomegalovirus (CMV) promoter, was destroyed (data not shown). In order to select the cells that have viral insertion in gene area and to quickly find the insertion site, we adopted a poly(A) trapping strategy (33). Poly(A) trapping was selected over promoter trapping because the marker gene expression in poly(A) trapping does not depend on the activity of the endogenous promoter, and 3′-RACE performed after poly(A) trapping to identify the disrupted gene is much easier to apply than 5′-RACE used after promoter trapping. Previously, we have shown that the poly(A) trapping method was an effective and convenient approach (25,34,35).

Figure 2, C–E, shows the consequences of all possible retroviral insertions events. Since the expression of *blasticidin+* gene is dependent on the downstream poly(A) signal sequence from the endogenous gene, blasticidin resistance can be used to select the cells that had a viral insertion in genes (either in an intron or exon). The inserted ribozyme will cleave the transcript initiated by the promoter of a disrupted gene. Eighteen blasticidin-resistant clones, resulting from viral infection, were analyzed by Northern blot analysis. Only one *blasticidin+* transcript with different sizes was found in each of the different clones (data not shown), indicating that only one gene disruption was occurred in most, if not all, of the blasticidin-resistant clones. This is consistent with a report that retroviral insertion numbers can be controlled at one per cell (36).

We infected 10^7 quasi-haploid cells with the retroviral vector described above and obtained approximately 9×10^3 blasticidin-resistant clones. The 9×10^3 balsticidin-resistant clones were pooled and plated at about 10,000 cells in a 15-cm plate in the presence of lethal toxin. After 6–7 doubling, 98 surviving clones were isolated and re-exposed to lethal toxin to confirm their lethal toxin resistance. Among them, 49 clones which retained lethal toxin resistance were selected for further studies.

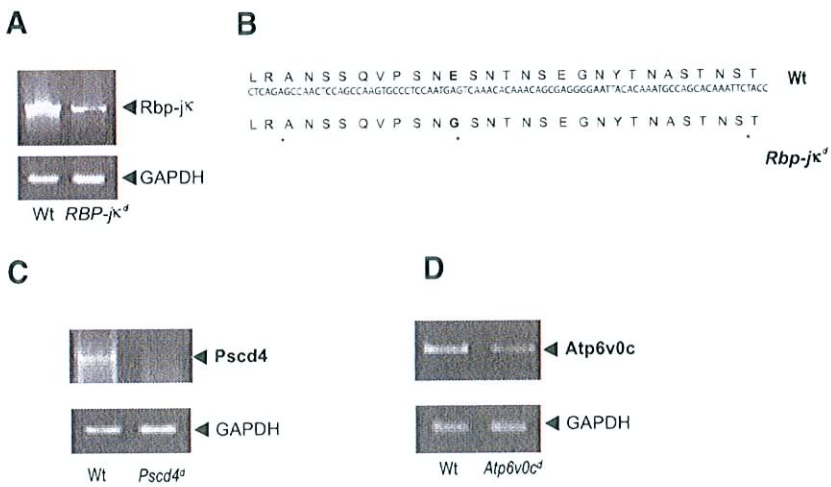

Figure 4. Analysis of the other allele of *Rbp-jκ*, *Pscd4*, *Atp6v0c* in their corresponding deficient cells. (A) Reverse transcription PCR (RT-PCR) was performed using a primer pairing with exon 1 sequence of *Rbp-jκ* and another targeting the sequence around stop codon of *Rbp-jκ*. The PCR product derived from messenger RNA (mRNA) of wild-type and *Rbp-jκ^d* cells were analyzed on agarose gel and sequenced. Arrows and stars shows the mutations sites. (B) RT-PCR was performed as in panel A using primers targeting the exon 1 and stop codon region of the *Pscd4* gene. The PCR products from wild-type and *Pscd4^d* cells were analyzed on an agarose gel. (C) Atp6v0c mRNA was amplified as in panel A using the RNA isolated from wild-type and *Atp6v0c^d* cells. The PCR products were analyzed on an agarose gel and sequenced. Rbp-jκ, J κ recombination signal binding protein; Pscd4, cytohesin-4; Atp6v0c, subunit c of vacuolar H+-ATPases; Wt, wild-type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

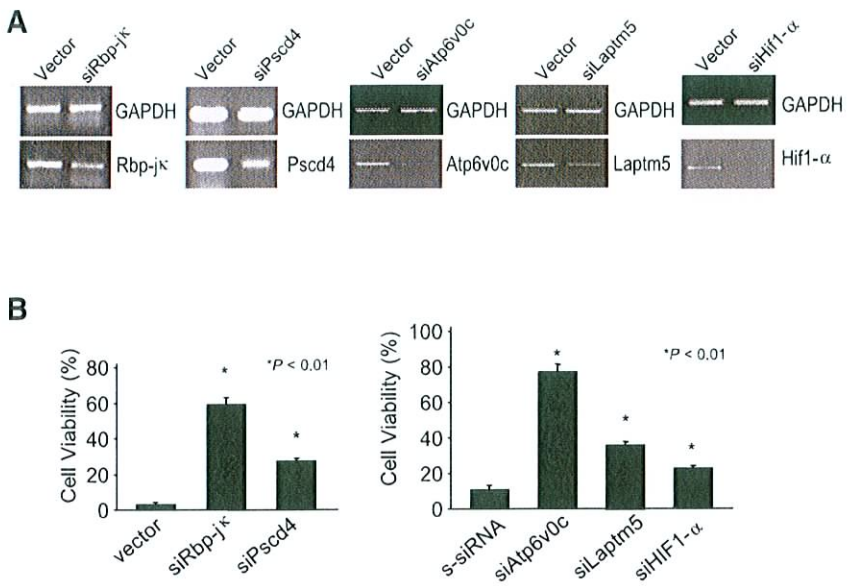

Figure 5. Conformation of the role of Rbp-jκ, Pscd4, Atp6v0c, Laptm5, and Hif1-α in lethal toxin-induced RAW 264.7 cells death. (A) Rbp-jκ or Pscd4 was knocked down by stable expression of targeting small interfering RNA (siRNA) (siRbp-jκ or siPscd4) in wild-type RAW 264.7 cells. Atp6v0c, Laptm5, and Hif1-α were knocked down by transfecting synthesized siRNAs targeting siAtp6v0c, Laptm5, or Hif1-α in wild-type RAW 264.7 cells. The reduction of these genes was determined by reverse transcription PCR (RT-PCR). (B) The Rbp-jκ, Pscd4, Atp6v0c, Laptm5, and Hif1-α knockdown cells together with the paralleled control cells (vector; for stable expression control) or scrambled siRNA transfected cells (s-siRNA; for synthesized siRNA transfection control) were treated with 500 ng/mL protective antigen (PA) plus 500 ng/mL lethal factor (LF), and cell viability was determined by crystal violet uptake. Rbp-jκ, J κ recombination signal binding protein; Pscd4, cytohesin-4; Atp6v0c, subunit c of vacuolar H+-ATPases; Laptm5, lysosomal-associated protein transmembrane 5; Hif1-α, hypoxia-inducible factor 1-α subunit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Since insertion of pDisrupt 8 in a gene will yield a fusion mRNA derived from the blasticidin resistance gene and the targeted gene (Figure 2), we used 3′-RACE to identify retrovirus-disrupted genes in the lethal toxin-resistant clones. The 3′-RACE products were sequenced, and the cDNA sequences fused with blasticidin-resistant gene cDNA were aligned with GenBank® database using Basic Local Alignment Search Tool (BLAST). Twenty-three different genes were identified. Some of them were identified more than once, suggesting duplications occurred in the process.

Figure 3 shows six examples of the junction sequences of the fused cDNA, showing the effectiveness of the poly(A) trap strategy. The viral insertion occurred in the genes of Rbp-jκ, cytohesin-4 (Pscd4), subunit c of vacuolar H+-ATPases (Atp6v0c), lysosomal-associated protein transmembrane 5 (Laptm5), hypothetical protein LOC68066, and Hif1-α. Rbp-jκ is a transcription factor recognizing a consensus sequence 5′-C(T)GTGGGAA-3′ and regulating viral and cellular gene transcription (37). Pscd4 is a member of cytohesin family, which is a guanine nucleotide exchange factor regulating the ADP-ribosylation factor of small GTPases (38). Atp6v0c is a subunit of a vacuolar proton pump, which is required for intracellular vacuoles acidification (39). Laptm5 is a lysosomal-associated multispanning membrane protein with unknown function (40). LOC68066 is a hypothetical protein homolog to human mitochondrial carrier CGI-69 (41). Hif1-α is a transcription factor that regulates the adaptive response to hypoxia in mammalian cells (42). By analyzing the sequence of fused cDNA, we predicted that splice donor element downstream from the *blasticidin+* sequence in pDisrupt 8 spliced to the splice acceptor of exon 2 of the *Rbp-jκ* gene, the splice acceptor of exon 7 of the *Psad4* gene, the splice acceptor of exon 2 of the *Atp6v0c* gene, the splice acceptor of exon 3 of the *Laptm5* gene, the splice acceptor of exon 2 of *LOC68066* gene, and the splice acceptor of exon 2 of *Hif1-α* gene, respectively (Figure 3).

Among them, the first three clones (referred as *Rbp-jκ^d*, *Pscd4^d*, and *Atp6v0c^d*, respectively) were further examined whether the other alleles of *Rbp-jκ*, *Pscd4*, and *Atp6v0c* were mutated in the corresponding cells. To specifically amplify the mRNA transcribed from the allele that was not hit by retrovirus, we amplified the cDNA region covering exon1 to the stop codon of *Rbp-jκ*, *Pscd4*, and *Atp6v0c* by RT-PCR. The same size PCR product was obtained from wild-type and *Rbp-jκ* cells (Figure 4A, top panel). The PCR products were recovered from agarose gel and sequenced. Three point mutations were found in the Rbp-jκ cDNA amplified from *Rbp-jκ^d* cell (Figure 4B). A change of E to G resulted from one of the point mutations. Pscd4 mRNA cannot be found in *Pscd4^d*cells (Figure 4C). Reported mutation spectrum showed that ENU cannot only result in amino acid substitution, but can also affect transcription. The undetectable Pscd4 expression suggests that either the transcription of Pscd4 from the other allele was impaired by chemical mutagen or is silent naturally in RAW 264.7 cells. RT-PCR and sequence analysis revealed that *Atp6v0c^d* cells had reduced Atp6v0c mRNA (Figure 4D) but no mutation in the coding region of the mRNA, indicating that the mutation occurred only on one allele in this cell, and *Atp6v0c* is a haploid insufficient gene in lethal toxin-induced cell death.

To confirm the genes identified by our method were indeed responsible for the lethal toxin-resistant phenotype, we used siRNA to knockdown the five genes identified in wild-type RAW 264.7 cells to see whether lethal toxin resistance can be reproduced. As shown in Figure 5, siRNA knockdown of all the six genes rendered resistance to lethal toxin-induced cell death in RAW 264.7 cells with varying degrees. The best protection from LeTx-induced cell death was detected in Atp6v0c knockdown cells. Atp6v0c is involved in the acidification of intracellular vacuoles (43). Since endosome acidifi-

cation is known to be required for lethal toxin function (44), a requirement of Atp6v0c in lethal toxin-induced killing of RAW 264.7 cells is anticipated.

DISCUSSION

To overcome the major impediments to the use of genetic screening in cultured cells, we have successfully developed a strategy using chemical mutagenesis to generate a quasi-haploid cell population that can then be used for insertional mutagenesis to create phenotypes. However, the fact that disruption of a given gene creates a phenotype within a quasi-haploid cell line must be interpreted with some qualifications. In certain instances, the gene of interest will be essential to the process under study in any genetic background (e.g., monogenic phenotypes in this case). In other instances, the phenotype will reflect epistatic interactions between non-allelic mutations induced by the chemical mutagen and the insertional mutation that is recovered. Whether the gene identified is responsible for a monogenic or polygenic phenotype can be determined by knockdown of the gene in wild-type cells using RNAi or another method. Although monogenic phenotype has been the principal object of study to date, polygenic phenotype is undoubtedly more common in nature and better explains the differences that exist between individuals in most populations. In some respects, our method addresses the question: "What mutations are essential for a biological function given the existence of other, unspecified mutations scattered throughout the genome?" A screen for such mutations, if carried to saturation, would reveal all or most of the genes that serve as the substrate for a defined, complex phenotype and the magnitude of the effect wrought by their destruction.

We have successfully obtained approximately 9×10^3 blasticidin-resistant clones from 10^7 quasi-haploid cells. The efficiency for generating the blasticidin-resistant clones was estimated to be about 1% of the cells infected with virus (approximately 2% insertion occurred in gene region, and only one orientation of viral insertion in gene region lead to the expression of resistant gene). Since some gene areas are transcribed in both directions and cryptic poly(A) sites could be utilized by the blasticidin transcript, the actual number of genes targeted by the retroviral vector could be an order of magnitude less than the number of apparent blasticidin-resistant clones. Assuming that the retroviral insertion is random, approximately 2.5%–25% of genes ($9 \times 10^{2-3}$ out of 3.5×10^4 of total genes) has been mutated once, and approximately 0.15%–1.5% (12% × 50%) hits should result in double allele mutations. The rate of biallelic mutation using this mutagenesis is superior to that generated by LOH in *Blm*-deficient ES cells (0.01%–0.1%) (21,22). In order to have all genes being mutated on their both alleles, $2.3 \times 10^{7-8}$ ($3.5 \times 10^4 \div 0.15$ to 1.5%) blasticidin-resistant clones are required, which is clearly feasible.

There are still limitations of this method. This approach cannot be used to screen the genes that are essential for cell survival in culture. Multiple rounds of chemical mutagen treatments could also lead to unpredictable induction of polyploidy and/or aneuploidy and selection of tolerant cells to chemical mutagen-induced damages, which can obscure the effects of the subsequent insertional mutagenesis. The plasticity of cultured cells is a problem associated with the use of genetic approaches in cultured cells, and this may be compounded by increased genome instability after high-intensity chemical mutagenesis. At present, the most effective way to minimize plasticity in cultured cells is to use the cells that have as few passages number as possible. In principle, the genetic approach described in this report only can be used to investigate aspects of biology that can be recapitulated in a cell culture model. However, the concept used in designing this approach may be extended to germline mutagenesis. A certain level of haploidy can be found in mice with chromosome deletions. Millions of genetic differences between different mice strains are haploid in the hybrid offspring. Applying insertional mutagenesis in the ES cells from these mice may facilitate phenotype screens in ES cells and may be useful in studies of certain complex phenotypes.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Address correspondence to Sung O. Kim, Department of Microbiology and Immunology, University of Western Ontario, London, ON, N6G 2V4, Canada. e-mail: sung.kim@schulich.uwo.ca

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Isolation and Characterization of a Near-Haploid Human Cell Line

Maciej Kotecki,[1,2] P. Sanjeeva Reddy,[2,3] and Brent H. Cochran[4]

Department of Physiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, Massachusetts 02111

Mammalian somatic cells are usually diploid. Occasional rare human tumors have been shown to have a hypodiploid karyotype. We have isolated a near-haploid subclone (P1-55) from a heterogeneous human leukemia cell line, KBM-7. These near-haploid cells have approximately half the human diploid DNA content and have a haploid karyotype except for a disomy of chromosome 8 (25, XY, +8, Ph$^+$). This cell line maintains a majority of cells with a near-haploid karyotype for at least 12 weeks in culture. By serial subcloning, we have isolated near-haploid subclones that maintain ploidy for at least 8 months in culture. Near-haploid cells can also be efficiently isolated from mixed ploidy cultures by size selection. The availability of this human near-haploid cell line should facilitate the genetic analysis of cultured human cells. © 1999 Academic Press

Key Words: **haploidy; KBM-7; somatic cell genetics.**

INTRODUCTION

One factor limiting the genetic analysis of mammalian cells in culture is that these cells have a diploid or higher number of chromosomes. As a result, the phenotype of a loss of function mutation on one chromosome is not expressed unless the corresponding allele on the other chromosome is also mutated. In contrast, bacteria and yeast are readily amenable to genetic analysis in part because they are haploid [1]. Previously, karyotypically stable cell lines which maintain a near-haploid karyotype have been isolated from amphibians and insects [2, 3]. There are currently no similar cell lines available for mammals. However, near-haploid karyotypes have been documented in rare human tumors and leukemias [4]. Thus, it seems that near-haploid mammalian cells can be viable.

Previously, Andersson *et al.* [5] established a heterogeneous (mixed ploidy) cell line (KBM-7) from the bone marrow of a patient with a near-haploid chronic myeloid leukemia. Though these cultures were initially slightly greater than 50% near-haploid, cells with a diploid or greater DNA content rapidly overgrow the KBM-7 cultures, rendering this cell line unsuitable for somatic cell genetics.

The karyotype of KBM-7 indicated that the near-haploid cells in the culture had disomies for both chromosomes 8 and 15 [5, 6]. However, leukemic cells isolated directly from this patient had two distinct near-haploid karyotypes. Some cells had disomies of both chromosomes 8 and 15, others had only a disomy of 8. Analysis of the leukemic cells from the patient also indicated the presence of a large percentage of near diploid cells with a karyotypic duplication of the 26, XY, +8, +15 near-haploid clone [5]. However, there were no near-diploid cells karyotyped that represented a duplication of the haploid clone with only a disomic 8. This observation suggested that the +8 near-haploid clone might be more karyotypically stable than the +8, +15 clone found in KBM-7.

Here we report the isolation and characterization of a near-haploid cell line which has only a disomy of chromosome 8 from the heterogeneous human leukemia cell line KBM-7. This cell line remains karyotypically stable for many weeks in culture and near-haploid suclones can be repeatedly isolated from this population of cells, allowing for the continuous maintenance of near-haploid cells in culture. These properties should make this cell line useful for somatic cell genetics.

MATERIALS AND METHODS

Cell culture and DNA content analysis. KBM-7 and its derivatives were routinely cultured in Iscove's medium + 15% fetal calf serum at 37°C in an atmosphere of 5–10% CO_2. Cultures were passaged every 3–5 days at a density not less than 5×10^5 cells/ml. For DNA content analysis, cells were stained with 9 μM Hoechst 33342 (Molecular Probes, Eugene, OR) and 0.3 μg/ml Dio-C5-3(3,3-dipentyloxa-carbocyamine) in culture medium at 37°C for 60 min and the DNA content per cell was estimated by measuring the fluorescence using a UV laser at 70–200 mW power in a Becton Dickinson FACStar Plus cell sorter [7].

[1] Permanent address: Institute of Human Genetics, Polish Academy of Sciences, Poznan, Poland.
[2] These authors contributed equally to the work presented in this report.
[3] Current address: Axys Pharmaceuticals, 11099 North Torrey Pines Road, La Jolla, CA 92037.
[4] To whom correspondence and reprint requests should be addressed. Fax: 617-636-6745. E-mail: cochran@opal.tufts.edu.

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Cell cloning. Haploid cell clones were isolated by cloning in soft agar. First, a 5-ml bottom layer of 0.8% agarose in Iscove's medium containing 15% fetal calf serum was poured into 60-mm dishes. Then the KBM-7 cells (100–10,000 cells) were mixed with 5 ml of medium containing 0.37% low-melting-point (LMP) agarose at 37°C and overlaid on top of the bottom agarose layer. The LMP agarose layer was rapidly solidified by placing ethanol-soaked paper towels on the plates for 15 min. The plates and the paper towels were separated by aluminum foil. Then the plates were incubated at 37°C and 5–10% CO_2 for 2–3 weeks. Cells were fed by overlaying 4 ml of medium in 0.37% LMP agarose every 7 days. When the colony size reached approximately 1 mm in diameter, colonies were picked and expanded in 1 ml medium in a 24-well plate and the haploid clones were identified by FACS analysis after 2–4 weeks of culture.

Karyotyping. For karyotyping, P1-55 cells 12–14 weeks after subcloning were harvested following incubation with 60 μg/ml bisbenzimid (Sigma Chemical Co., St. Louis, MO) and 2.5×10^{-5} M ethidium bromide (Sigma Chemical Co.) for 2.5 h and 0.07 μg/ml colcemid (Gibco) for 1 h. Cells were then swelled in 0.56% KCl at room temperature for 8 min. Cells were pelleted and a −20°C mixture of glacial acetic acid and methanol 1:3 (v/v) was added dropwise without agitation in order to prevent chromosomal losses resulting from metaphase plate rupture and overspreading. Agitation was found to preferentially rupture the near-haploid metaphases. Chromosomes were GTG-banded as described [8].

RESULTS

Isolation of P1-55. Consistent with the work of Andersson *et al.* [5], we found the partially near-haploid heterogeneous KBM-7 cell line to be highly karyotypically unstable. First-passage cultures from this cell line show that approximately 55% of the cells have a near-haploid DNA content by FACS analysis (see Fig. 1A). However, when these cultures are further passaged, cells with a diploid or greater DNA content quickly overgrow the culture (see Fig. 1B). This karyotypic instability renders the KBM-7 cell line unsuitable for somatic cell genetics.

To determine whether more stable near-haploid cells could be isolated from the KBM-7 cultures, first-passage KBM-7 cells were subcloned in soft agar. Sixty-two subclones were isolated and characterized by FACS analysis for DNA content. Of these 62 cell lines, five showed a near-haploid DNA content. The percentage of clones that were near-haploid was less than their representation in the starting population because near-haploid cells were found to have a lower cloning efficiency in soft agar than near-diploid cells (data not shown). Three of these haploid subclones quickly evolved into higher ploidies upon further cultivation. The most stable of the near-haploid subclones, which we call P1-55, maintains a near-haploid DNA content in the majority of cells for up to 12 weeks in culture. The G1 DNA content of this cell line was 51 ± 2% of second-passage G1 human primary fibroblasts as measured by Hoechst 33342 dye staining (Fig. 1C). By the same method, the G1 DNA content of the haploid cells in the KBM-7 line was within 2% of the DNA content of G1 phase P1-55 cells. P1-55 DNA content determined by comparison to primary human lymphocytes was slightly higher–approximately 60%. The difference is likely due to differential dye uptake and stainability of growing fibroblasts and quiescent lymphocytes. The expected value based on the karyotype (see below) would be 53% [9].

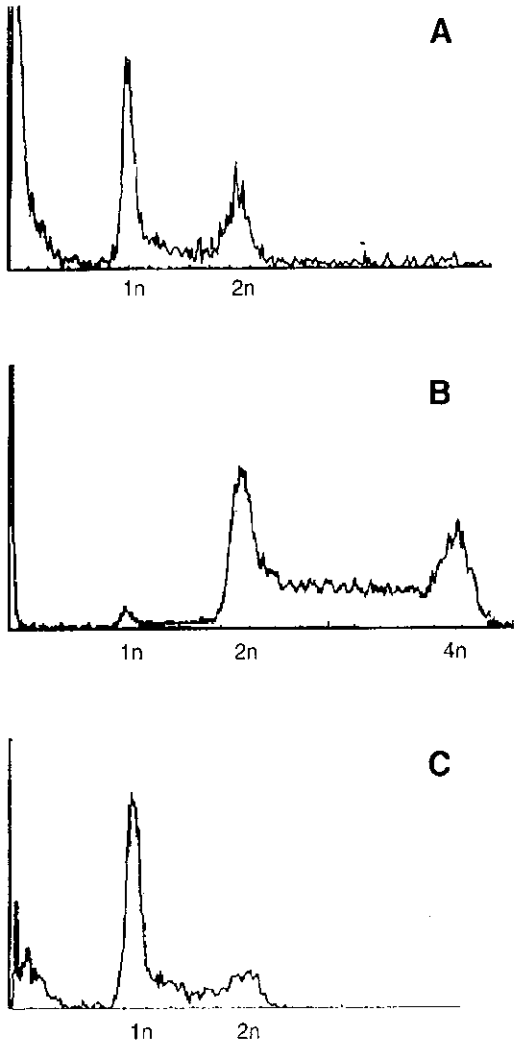

FIG. 1. Estimation of ploidy and DNA content of KBM-7 and the near-haploid clone P1-55 by flow cytometric analysis. Cells were analyzed for DNA content as described under Materials and Methods. Fluorescence intensity scales varied slightly between experiments. Human primary fibroblasts were used as standard to determine the relative fluorescence intensities of $1n$, $2n$, and $4n$ quantities of the human haploid DNA content. The positions of the $1n$, $2n$, and $4n$ peaks are indicated. (A) KBM-7 at first passage. (B) KBM-7 after 20 days in culture. (C) The near-haploid subclone P1-55 at 4 weeks after subcloning from soft agar.

Karyotype. At 12 to 14 weeks in culture after subcloning, cells from the P1-55 cell line were analyzed for karyotypes. A typical near-haploid karyotype is shown

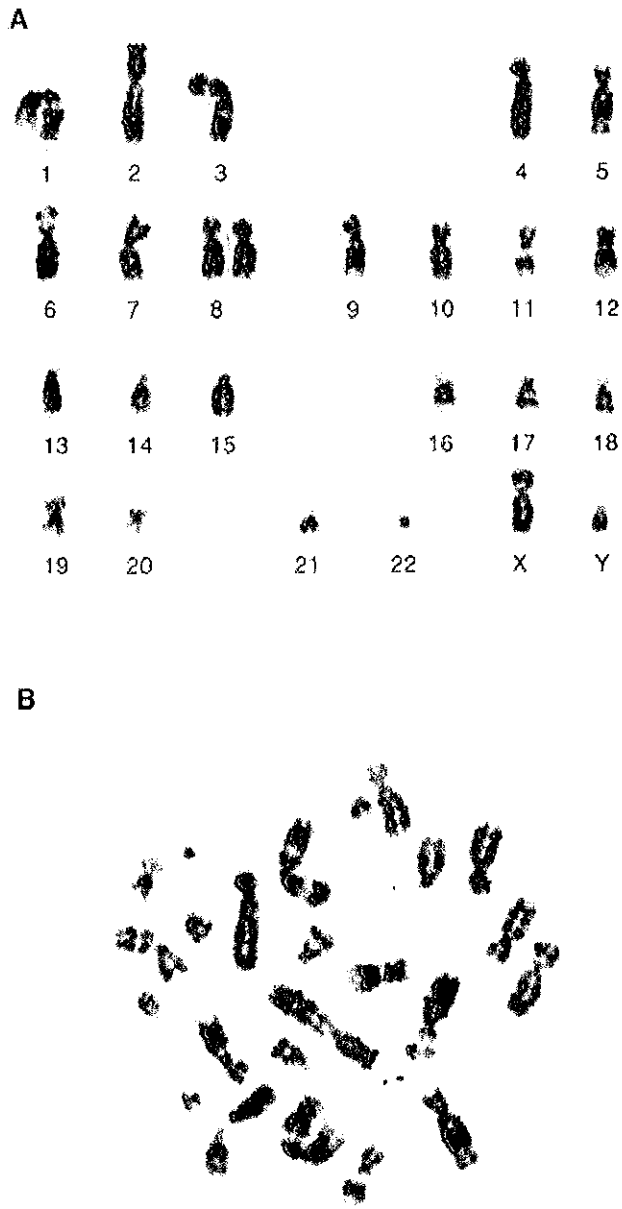

FIG. 2. Karyotype of the near-haploid clone P1-55. The P1-55 cell line was karyotyped as described under Materials and Methods. (A) A representative karyotype showing disomy of chromosome 8 and the t(9q;22q) Philadelphia chromosome in a cell with 25 chromosomes. (B) A near-haploid cell in metaphase.

in Fig. 2. A summary of these karyotypes is given in Table 1. The most frequent karyotype observed was that of cells with 25 chromosomes with monosomies at every chromosome except chromosome 8, which was disomic (25, XY, +8, Ph^-). A variation of this karyotype was also found that was identical except that it lacked the Y chromosome, which is frequently lost in leukemic cells [10]. Except for the Ph translocation t(9q;22q) which was present in all cells, no structural rearrangements involving two chromosome breakpoints in the same metaphase were found in the P1-55 cells. The P1-55 karyotype is concordant with the presumably more stable near-haploid cells found in bone marrow cells of the patient from which KBM-7 was derived, but differs from the one reported for KBM-7 itself by the monosomy for chromosome 15 and the presence of the Y chromosome [5]. Thus, it is likely that the cells with only a disomy for chromosome 8 were a small fraction of the first-passage KBM-7 population and were rapidly overgrown by the karyotypically less stable 26, XY, +8, +15 clone (see discussion below and Fig. 3).

The karyotype analysis also reveals that the P1-55 cell line is not homogeneous with respect to ploidy. Several near-diploid cells were found which were apparent duplications of the near-haploid clones with disomic Philadelphia chromosomes and tetrasomies of chromosome 8 (Table 1). In addition, some near-tetraploid metaphases were observed. Two of these cells were karyotyped and were found to be almost exact karyotypic duplications of the diploid cells. Consistent with the FACS analysis, no clonal karyotypes were found to have intermediate numbers of chromosomes between 1, 2, and 4*n*. Because these near-diploid and near-tetraploid cells have karyotypes that are even multiples of the predominate haploid cells, it is likely that they were formed in a single event by either endoreduplication or cell fusion rather than gradually. Since no triploid cells were observed, it is unlikely that either the diploid or tetraploid cells arose by cell fusion.

SSR polymorphism analysis. The near-haploid karyotype of P1-55 does not imply that every genetic locus not on chromosome 8 is haploid, since small duplications and translocations cannot be identified at this level of resolution. Nevertheless, since the overall DNA content of these cells is ~52% of human diploid fibroblasts, the great majority of loci are likely to be haploid. Simple sequence (CA) repeat polymorphisms were examined for several arbitrarily selected chromosomes and chromosome 8, to determine whether a molecular analysis of zygosity would be consistent with the near-haploid karyotype and whether the disomic chromosome 8 was homo- or heterozygous. (See Table 2.) None of the loci tested on chromosomes 3, 10, and 15 from this cell line were found to be heterozygous. This result is consistent with the karyotype showing that these chromosomes were monosomic. In contrast, all (4/4) of the chromosome 8 loci tested were heterozygous. This result also indicates that the disomy of chromosome 8 did not arise by chromosome duplication. Loci on other chromosomes were not examined.

Stability. Although the P1-55 cell line was clonally derived, with increasing time in continuous culture, the percentage of the cells that had a duplicated set of

TABLE 1

Karyotype Analysis of the P1-55 Cell Line

No. of cells	No. of chromosomes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
8	24	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
50	25	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6	46	2	2	2	2	2	2	2	4	2	2	2	2	1	2	2	2	2	2	2	2	2	2	1	0
5	49	2	2	2	2	2	2	2	4	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	2
7	50	2	2	2	2	2	2	2	4	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
1	98	4	4	4	4	4	4	3	7	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
1	98	4	4	4	4	4	4	4	7	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	4

Note. Karyotypes were determined by GTG banding of P1-55 as described under Materials and Methods. One hundred thirty-one metaphases with good spreading and banding patterns were photographed and karyotyped. A karyotype was considered to be clonal within the population if at least five cells were observed with the same karyotype. Unique karyotypes were found for 43 metaphases with chromosome numbers ranging from 22 to 49. The random distribution of chromosome losses (except for preferential loss of X and Y) in these spreads likely indicates that these karyotypes were not clonal populations, but were more likely generated by rupture of near-diploid metaphase plates during preparation. Since only high-quality chromosome spreads were selected for complete analysis, table does not quantitatively reflect the relative distribution of near-haploid and near-diploid cells in the culture. In addition, the quantitative distribution of cell karyotypes is further skewed by the fact that optimal fixation conditions differed for near-haploid and near-diploid cells.

chromosomes increased. (See Fig. 3.) However, even after 12 weeks in culture, more than 50% of the cells had a near-haploid DNA content. This number is actually an underestimate of the percentage of the haploid cells in the population since haploid cells in G2/M cannot be distinguished from diploid cells in G1 by DNA content measurement in the FACS. Nevertheless, the near-diploid cells must have a slight growth advantage over the near-haploid ones. However, since the majority of cells remain haploid for long periods of time in culture, the incidence of spontaneous increase in ploidy in these cultures must be relatively low. In addition, we have found that culture conditions and subcultivation protocols can affect the rate of diploid outgrowth, but even under identical cultivation protocols other subclones of KBM-7 that were examined evolved into higher ploidies much more quickly than P1-55. The least stable of these (P1-32) was as unstable as KBM-7 and was found to be disomic for both chromosomes 8 and 15 and lacked Y as was reported for the original KBM-7 line [5] and the KBM-7/B5 subclone [6]. (See Fig. 3.) This cell line also had a faster doubling time than P1-55 (data not shown). These properties of P1-32 could account for the rapid diploidization of KBM-7 and the predominance of cells with the +8, +15 karyotype in the KBM-7 line.

TABLE 2

Heterozygosity Analysis of P1-55 Using Simple Sequence Repeat Polymorphisms

	Zygosity (heterozygous/number loci tested)		
Chromosome No.	KBM-7	P1-55	control
3	0/4	0/4	2/4
8	4/4	4/4	3/4
10	0/4	0/4	4/4
15	2/4	0/4	4/4

Note. Markers of CA repeat polymorphisms from the indicated chromosomes were analyzed as described by Hudson *et al.* [31]. HeLa cell DNA was used as a control for chromosome 3 markers and CEPH cell DNA was used as a control for all the chromosome polymorphisms examined. Loci examined for polymorphisms were as follows: chromosome 3—D3S 1209, D3S 1212, D3S 1215, D3S 1216; chromosome 8—D8S205, D8S206, D8S207, D8S208; chromosome 10—D10S172, D10S173, D10S174, D10S175; chromosome 15—D15S98, D15S100, D15S101, D15S102. The probability of detecting polymorphisms on any one of the analyzed chromosomes for a given human diploid cell with these markers is >99%.

Despite the tendency to drift toward higher ploidies, haploid subclones (P1-55-S1) could be reisolated from the mixed-ploidy cultures of P1-55 and maintained in culture for long periods with a high percentage of near-haploid cells (Fig. 3). Further subcloning of P1-55-S1 has yielded haploid cells with even better stability in culture as shown in Fig. 3B. These cells can be subcloned in soft agar with an efficiency of 10–15%. Some of these S2 subclones have been maintained as principally haploid cultures for as long as 8 months and have a normal doubling time of 24 h. Thus, cultures of near-haploid P1-55 cells can be maintained indefinitely.

Work on haploid frog embyos indicated that the haploid cells were smaller than their diploid counterparts [11]. To determine whether this is also true of this cell line, populations of near-haploid and diploid subclones were mixed and the smaller cells in this population were sorted by light scattering. Figure 4 shows that the vast majority of small cells have a near-haploid DNA

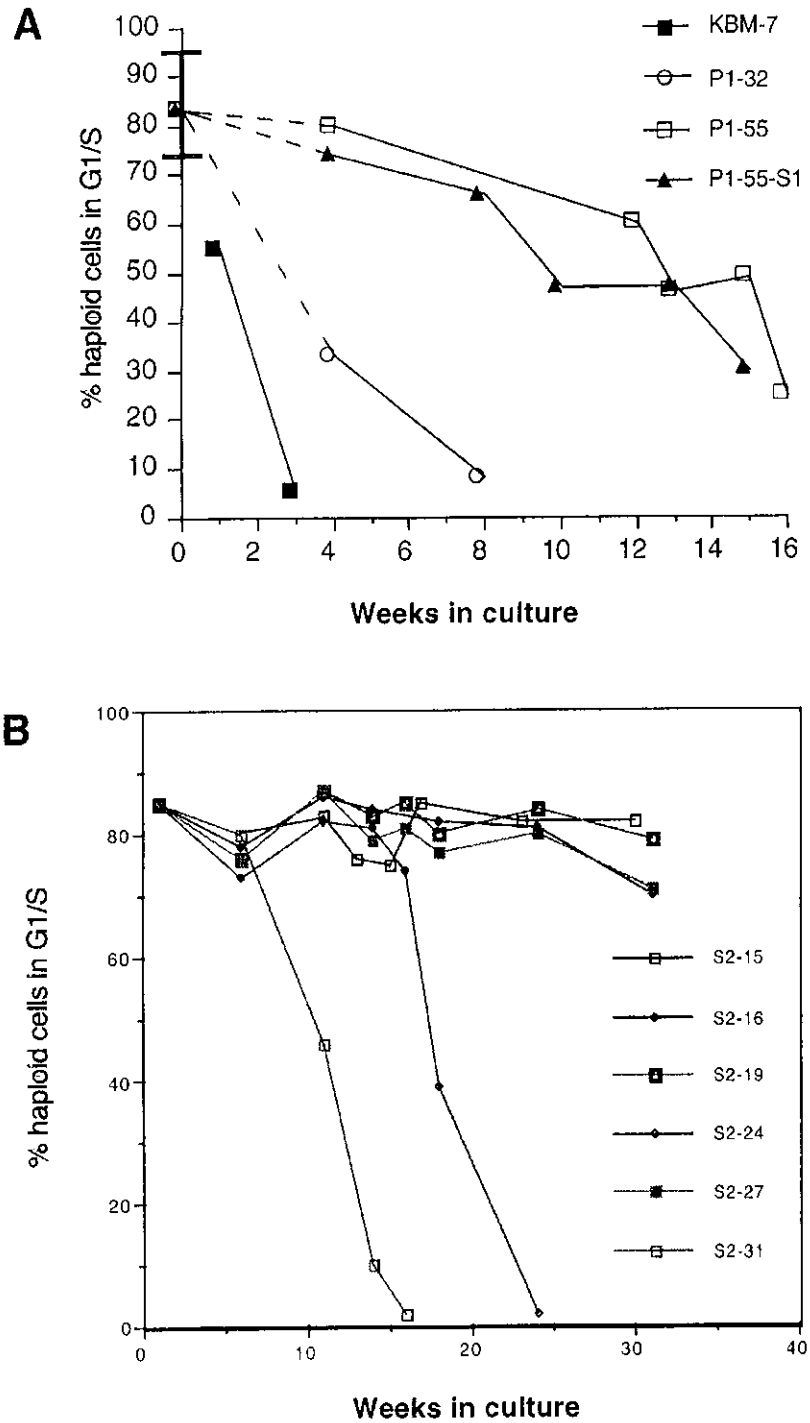

FIG. 3. DNA content change of near-haploid cell lines as a function of time in culture. (A) Subclones P1-32 and P1-55 of the KBM-7 cell line were isolated by cloning in soft agar. P1-55-S1 was isolated by subcloning P1-55. The DNA content of these cell lines was determined by FACS analysis at the indicated numbers of weeks in culture by the methods described under Materials and Methods. The percentage of haploid cells in G0/G1 and S was estimated either by using the MODFIT program (Verity Software House) or by direct peak area measurement. The percentage of haploid cells in G2 and M at any given time point could not be precisely determined because of the overlapping diploid cell peak in G0/G1. Twenty-one independent measurements of populations of haploid cells with no $4n$ peaks (no detectable diploid cells) indicate that the percentage of haploid cells in G1 + S at any time in culture ranges from 73 to 94% with a mean of 83%. The zero time point for the cloned cells begins with the day the cells were isolated from soft agar. At this time, too few cells existed to analyze by FACS. Assuming the cells were purely haploid at that time, approximately 83% would be in G1 + S. The dashed lines indicate this assumption for the zero time point. Since KBM-7 was not clonal, this assumption was not made. All other data points are actual measurements. The relative and absolute stability of each of the subclones remained consistent through multiple trials. Representative experiments are shown. (B) Stability of second-generation subclones (S2) of near-haploid P1-55 cells obtained after a second round of subcloning in soft agar was estimated for DNA content using flow cytometry.

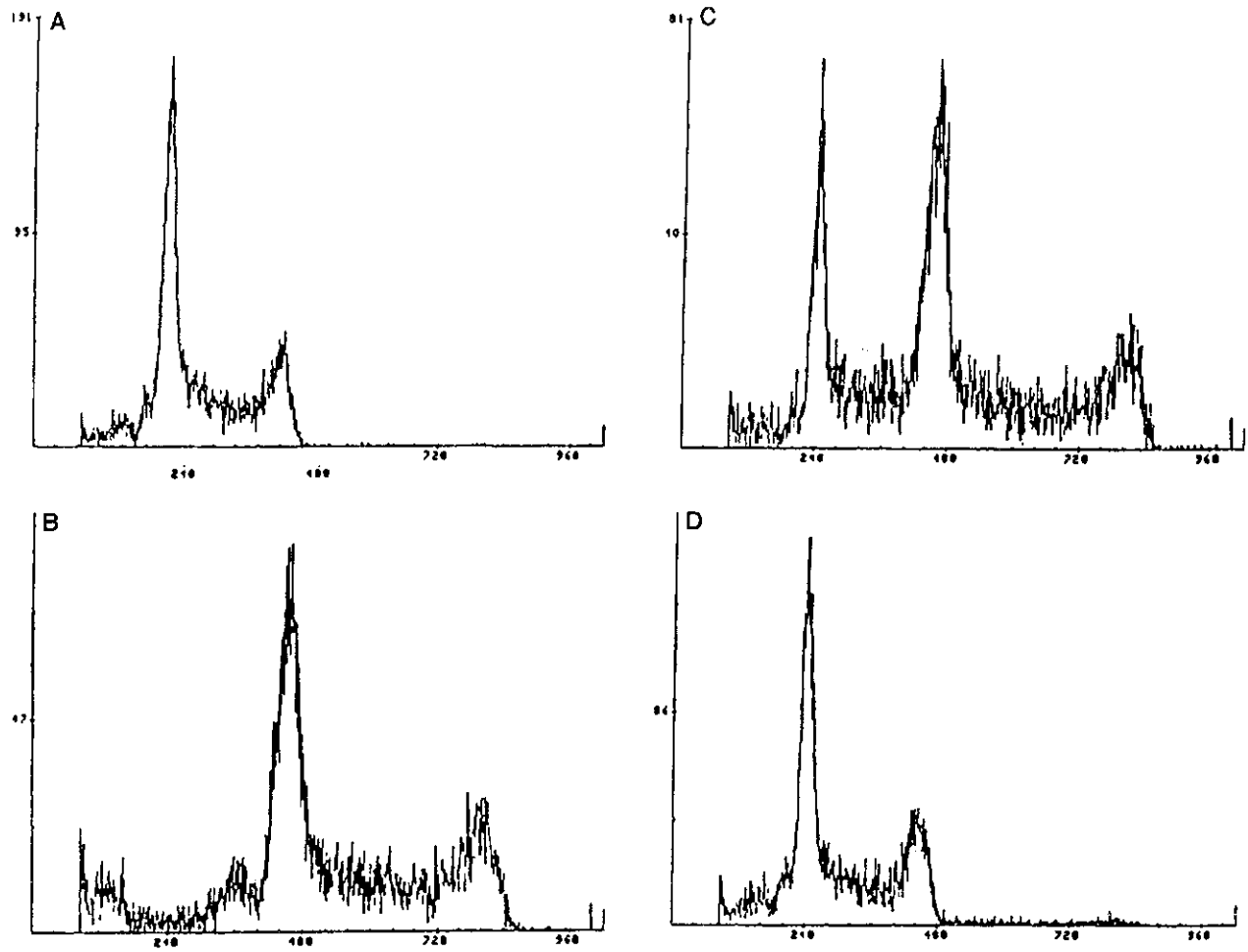

FIG. 4. Sorting of haploid cells based on size. A mixture (C) of haploid (A) and diploid (B) cells was sorted for the smallest cells using forward and side scatter analysis in flow cell sorter without any staining. Each histogram shows analysis of DNA content of a population of viable cells stained with Hoechst 33342. (A) DNA content of haploid clone S2-19. (B) DNA content of diploid clone S2-8. (C) DNA content of haploid S2-19 and diploid S2-8 cells mixed (#/# 1:1) right before size-sorting. (D) DNA content of smaller cells sorted out of the mixture of haploid and diploid cells (shown in C).

content. Thus, this technique can be used to isolate haploid cells from mixed ploidy populations.

DISCUSSION

The existence of the P1-55 cell line demonstrates that full diploidy is not required for the maintenance of human cells in culture. In fact, the existence of this cell line demonstrates that every chromosome except perhaps chromosome 8 can exist as a monosome in culture. These cells are obviously not normal since they have the Philadelphia translocation in addition to the change in ploidy. However, they do have characteristics typical of many other mammalian cell lines such as a requirement for fetal calf serum for growth and the ability to maintain an exponential doubling time of approximately 12–30 h depending on the culture density (data not shown). From these observations, it can be inferred that if there are genes required in more than one copy for growth of human cells in culture, these must be a small percentage of the human genome.

The reasons for the maintenance of the disomy of chromosome 8 are unclear. It could be that there are genes on chromosome 8 that are required in two copies for the growth of the cells. Alternatively, there may be a gene on chromosome 8 that contributes to the establishment of the tumor or cell line (such as the *myc* gene) when maintained in multiple copies by the presence of the disomy. Yet another possibility is that there are independent recessive cell lethal mutations on both

homologues of chromosome 8 that are mutually complemented by the presence of the corresponding wild-type alleles on the other copy of 8. This would be consistent with the finding that there is heterozygosity at chromosome 8 in this cell line. We favor one of the latter two hypotheses since previous analyses of near-haploid leukemias do not always show disomic chromosome 8 [12, 13].

The mechanism by which these cells became haploid is unclear. It is possibly the result of an abnormal karyokinesis induced by chemotherapeutic agents used to treat this patient. Alternatively, specific genetic alterations may have contributed to the haploidization event. In yeast, a gene has been described that has the phenotype of frequent mitotic haploidization [14]. Perhaps a similar gene is mutated in these cells.

Despite their unusual karyotype, haploid vertebrate cells are likely to be capable of exhibiting a wide range of cellular responses. Haploid fish and amphibians though not viable show a significant degree of morphological and cellular differentiation and in some cases can live for several weeks [15–18] Moreover, haploid-euploid mosaic chickens are not only viable, but show representation of haploid cell lineages in a variety of tissues [19]. Thus, haploid cells could serve as good models of their diploid counterparts for a variety of biological phenomena.

The ability to isolate recessive mutations at a relative high frequency from Chinese hamster ovary (CHO) cells has led to the suggestion that the CHO cell genome is functionally hemizygous [20]. However, analysis of electrophoretic shift variants of CHO is consistent with the near-diploid karyotype of this cell line [21, 22]. The high rate of recessive mutant isolation is more likely due to a high frequency of deletion and chromosome loss uncovering relatively rare single gene mutations [23–25]. The advantage of a truly haploid cell line such as P1-55 is that there would be no need for secondary events to occur, thus increasing the relative frequency of phenotypic expression of recessive traits. In addition, it is not known with any certainty what percentage of the CHO cell genome is available for high-frequency isolation of recessive mutants, whereas in P1-55 virtually all loci not on chromosome 8 should be inactivated with single hit kinetics.

Thus, it should prove possible to use the P1-55 cell line and its derivatives to facilitate the application of somatic cell genetics to the study of mammalian cell biology. For instance, insertional mutagenesis of these cells by high-titer retroviruses should inactivate genes with a single hit, leaving the inactivated gene tagged with retroviral DNA and thereby facilitating the recovery of the affected loci [26]. Furthermore, use of vectors designed for homologous recombination in somatic cells has proved to be very useful for the analysis of diploid cultured cells [27–29]. Homologous recombination with these near-haploid cells would allow for the immediate expression of the phenotype of inactivated genes without the necessity of knocking out two alleles or passaging the gene knockout through chimeric animals [30]. The fact that these cells have a tendency to increase in ploidy with time in culture should not undermine the effectiveness of these genetic strategies since any genetic alteration that occurs in a haploid cell will presumably be duplicated and remain homozygous as diploidization occurs. Moreover, we have been able to reisolate near-haploid cells from mixed ploidy populations by both size sorting and subcloning. Therefore, these near-haploid cells provide several advantages over diploid ones for somatic cell genetics.

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Haploid Genetic Screens in Human Cells Identify Host Factors Used by Pathogens

Jan E. Carette, *et al.*

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Haploid Genetic Screens in Human Cells Identify Host Factors Used by Pathogens

Jan E. Carette,[1] Carla P. Guimaraes,[1] Malini Varadarajan,[1] Annie S. Park,[1] Irene Wuethrich,[1] Alzbeta Godarova,[1] Maciej Kotecki,[2] Brent H. Cochran,[2] Eric Spooner,[1] Hidde L. Ploegh,[1,3] Thijn R. Brummelkamp[1]*

Loss-of-function genetic screens in model organisms have elucidated numerous biological processes, but the diploid genome of mammalian cells has precluded large-scale gene disruption. We used insertional mutagenesis to develop a screening method to generate null alleles in a human cell line haploid for all chromosomes except chromosome 8. Using this approach, we identified host factors essential for infection with influenza and genes encoding important elements of the biosynthetic pathway of diphthamide, which are required for the cytotoxic effects of diphtheria toxin and exotoxin A. We also identified genes needed for the action of cytolethal distending toxin, including a cell-surface protein that interacts with the toxin. This approach has both conceptual and practical parallels with genetic approaches in haploid yeast.

Identification of gene products that play a role in human disease drives much of today's biomedical research. Classical genetics with induced mutations, as pioneered by Muller in 1927 (*1*), is the most powerful unbiased approach to elucidate the genetic components that underlie biological processes. The study of cultured human cells allows the recapitulation of many essential elements of human disease. However, the inability to efficiently generate and recover bi-allelic mutants in human diploid cells limits the contribution of mutagenesis-based genetics to the understanding of human disease.

The identification of cellular genes exploited by bacteria and viruses is essential to elucidate the mechanisms by which these pathogens cause disease (*2*–*4*). Bacterial toxins contribute greatly to pathogenicity of the microbes that produce them. Identification of host proteins involved in toxin cytotoxicity should help to identify targets for therapeutic intervention in diseases caused by bacteria, many of which now show increased resistance to conventional antibiotics. Likewise, an understanding of how viruses depend on host proteins to enter the cell, replicate their genome, and spread may accelerate the development of antiviral drugs. Influenza virus remains a threat to human health, causing several hundred thousands of deaths annually and many more in the course of a pandemic (*5*). The rapid spread of new strains of influenza A [for instance, avian (H5N1) and swine (H1N1) influenza] and the emergence of drug-resistant influenza strains (*6*) limit the effectiveness of vaccines and current antiviral therapeutics. Thus, we developed a method for genetic screens in human cells and isolated genes required for the action of several bacterial toxins and influenza viruses.

Development of an approach for haploid genetic screens in human cells. To facilitate mutagenesis-based genetic approaches in human cells, we used a derivative of the 7 KBM7 chronic myeloid leukemia (CML) cell line with a haploid karyotype except for chromosome 8 (Fig. 1A) (*7*, *8*). In this cell line of hematopoietic origin, gene inactivation should allow the generation of null mutants for most nonessential genes. We chose to inactivate genes with the use of insertional mutagenesis because this approach is highly mutagenic in a variety of organisms, and the integrated DNA sequences provide a convenient molecular tag to identify the disrupted gene. We used gene-trap retroviruses that contain a strong adenoviral splice acceptor site and a marker gene (green fluorescent protein or puromycin-resistance gene) in reverse orientation of the retroviral backbone. To examine whether gene-trap insertions were indeed mutagenic, we performed a pilot screen to isolate mutants resistant to the nucleotide analog 6-thioguanine (6-TG), converted to a toxic metabolite by the enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT). The gene-trap virus was titrated to obtain a single viral integration in the majority of the infected cells. Cell lines resistant to 6-TG were recovered, and five independent mutants carried insertions in intron 1 of the X-linked HPRT gene (Fig. 1B and fig. S1A). We next performed two genetic screens to target autosomal genes. KBM7 cells are sensitive to the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) and to inhibition of the BCR-ABL oncogenic fusion protein by the kinase inhibitor Gleevec (Novartis, Basel, Switzerland). Gene trap–mutagenized KBM7 cells were exposed to either TRAIL or Gleevec and

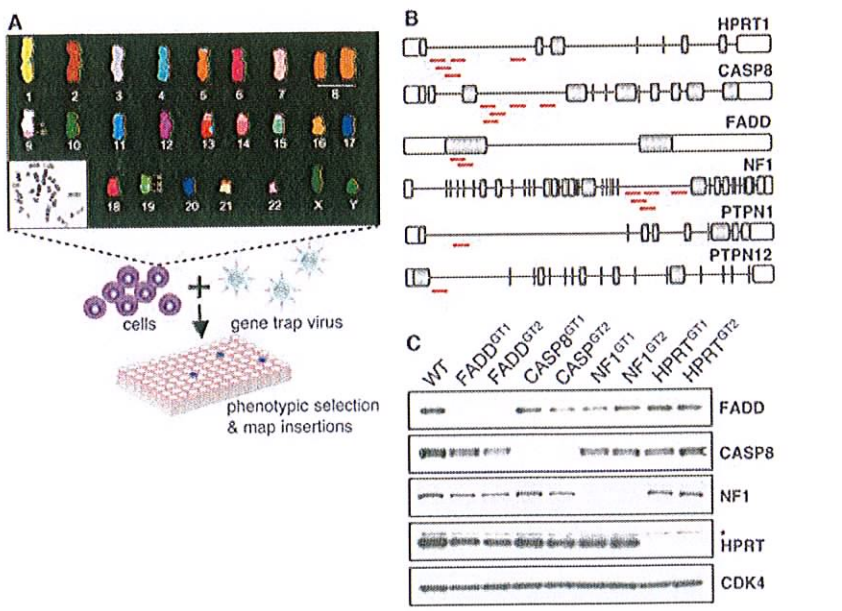

Fig. 1. Haploid genetic screens. **(A)** Twenty-four–color spectral karyotype of near-haploid KBM7 cells and schematic outline of gene trap–mutagenesis screens. **(B)** Schematic outline of the gene-trap insertion sites (red lines) in cells exposed to 6-TG, TRAIL, or Gleevec. **(C)** Immunoblot analysis of FADD, CASP8, NF1, and HPRT expression levels in clones that contain independent gene-trap insertions in the respective loci. Mutant alleles are labeled with GT in superscript notation, and an unspecific background band is indicated with an asterisk. CDK4 was used as a loading control.

[1]Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142, USA. [2]Department of Physiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA. [3]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142, USA.

*To whom correspondence should be addressed. E-mail: brummelkamp@wi.mit.edu

resistant mutants were recovered. Five TRAIL-resistant clones showed independent insertions in caspase 8 (CASP8) and two independent insertions in Fas-associated death domain protein (FADD), genes known to be required for TRAIL-induced apoptosis (Fig. 1B and fig. S1A) (*9*). Resistance to TRAIL was confirmed in these mutants (fig. S1B). Five independent Gleevec-resistant mutants contained insertions in neurofibromin 1 (NF1) and one in protein tyrosine phosphatase-N1 (PTPN1); both genes are known to play an important role in the response of CML cells to Gleevec (*10*). One insertion was found in PTPN12, a tyrosine phosphatase that interacts with c-abl and negatively regulates its activity (*11*). Thus, PTPN12 is critical for Gleevec sensitivity. All insertions were in the same transcriptional orientation as the target gene, and immunoblot analysis of HPRT, FADD, CASP8, and NF1 mutant cells failed to detect the corresponding gene products (Fig. 1C). The haploid background of KBM7 thus enables the generation of mutant alleles for autosomal genes and pinpoints genes involved in the biological processes under study.

Identification of host factors required for cytolethal distending toxin. Because many pathogenic agents such as bacterial toxins or viruses readily kill the cells they target, a large-scale production of knockout alleles for human genes may enable the identification of host factors essential for pathogenesis, such as enzymes that create structures recognized by toxins or viruses or the receptors themselves. Several pathogenic bacteria (such as *Escherichia coli*, *Shigella dysenteriae*, *Actinobacillus actinomycetemcomitans*, *Campylobacter jejuni*, *Helicobacter* spp., *Salmonella typhi*, and *Haemophilus ducreyi*) secrete potent bacterial toxins named cytolethal distending toxins (CDTs). The DNAse I–like CdtB subunit of these toxins enters the nucleus and causes cytotoxicity by inducing DNA breaks (*12*, *13*). The membrane receptor(s) and other essential host genes involved in the entry or action of CDTs are unknown. KBM7 cells responded to *E. coli*–derived CDT by undergoing an arrest in the G_2/M phase of the cell cycle (Fig. 2A) that precedes cell death. Mutagenized KBM7 cells were treated with CDT and resistant clones were isolated. Eleven independent insertions in sphingomyelin synthase 1 (SGMS1) and three insertions in TMEM181, a gene that encodes a putative G protein–coupled receptor (*14*), were recovered (Fig. 2B and fig. S2A). SGMS1 and TMEM181 mutants were resistant to CDT, a phenotype reverted by complementing the mutant cells with the corresponding cDNAs (Fig. 2C and fig. S2B). The SGMS1 mutation reduced levels of sphingomyelin, as verified by treatment of cells with lysenin, a sphingomyelin-specific pore-forming toxin (fig. S3A). Sphingomyelin is a key component of lipid rafts: Depletion of SGMS1 activity disturbs lipid-raft function and prevents receptor clustering (*15*), a trait of possible relevance for CDT binding and/or entry. Extraction of the lipid-raft component cholesterol from the plasma membrane abolishes CDT binding (*16*).

TMEM181 mutants remained fully sensitive to lysenin (fig. S3, A and B), suggesting that their resistance is acquired by means other than sphingomyelin depletion. Because TMEM181 is present at the cell surface (*17*) and a receptor for CDT must localize to the plasma membrane, we tested whether CDT bound to TMEM181. Flag-tagged CDT was adsorbed onto anti-Flag beads and incubated with cell lysates prepared from wild-type (WT) KBM7 cells and from KBM7 cells that express hemagglutinin (HA)–tagged TMEM181. Immunoblot analysis showed robust binding of TMEM181 to CDT (Fig. 2D). When TMEM181 was overexpressed by retroviral transduction in NIH3T3, U2OS, and Hela cells, it sensitized them to CDT intoxication (Fig. 2E and fig. S4), suggesting that TMEM181 expression levels are rate limiting for intoxication. Thus, CDT may bind to the cell-surface protein TMEM181, an event both required and rate limiting for intoxication, and then enter the cell through sphingomyelin-dependent, lipid-raft–mediated endocytosis, followed by nuclear entry and cleavage of cellular DNA. However, these results do not rule out the possibilities that TMEM181 is part of a complex that constitutes a functional receptor or plays a role in trafficking of a receptor-toxin complex.

Isolation of host factors essential for influenza virus infectivity. We next isolated mutant cells that were resistant to influenza virus A (PR/8/34; H1N1). Proviral-host junction sequencing revealed two independent insertions in cytidine monophosphate *N*-acetylneuraminic acid synthase (CMAS), encoding the enzyme responsible for activation of NeuAc to CMP-NeuAc, the glycosyl donor used in sialic acid–containing glycoconjugate synthesis. These structures are the receptors on influenza-susceptible cells recognized by the influenza hemagglutinin. We recovered three independent insertions in SLC35A2 (Fig. 3A and fig. S5A), a gene whose product transports uridine 5′-diphosphate–galactose from the cytoplasm to the Golgi, where it serves as a glycosyl donor (*18*) important for the generation of glycans to be modified with sialic acids. To determine whether mutant cells could be infected by influenza, we exposed cells to the virus and

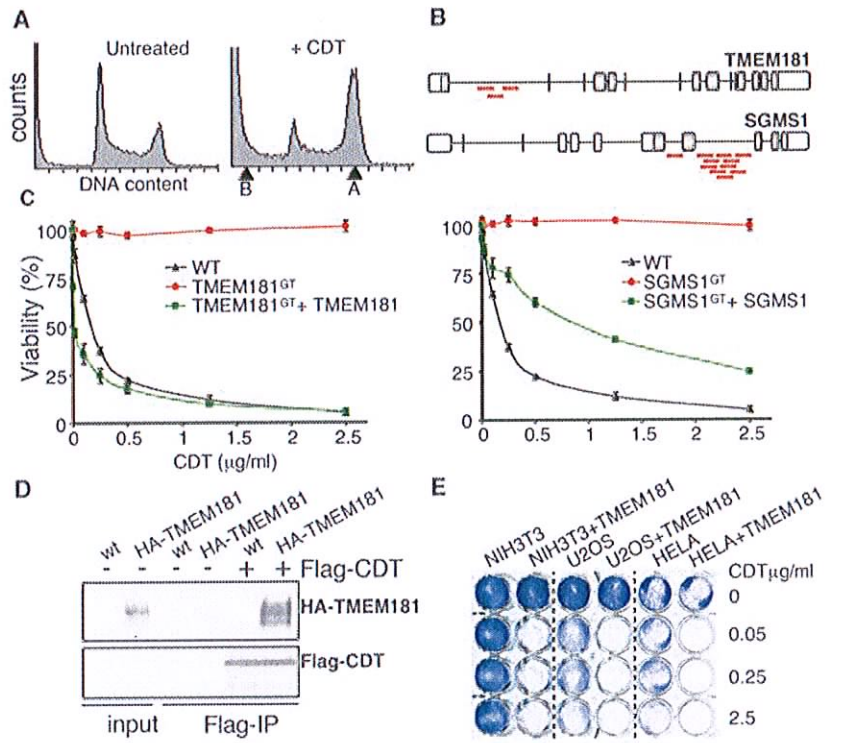

Fig. 2. Host factors for CDT. **(A)** Flow cytometric analysis of control KBM7 cells (left) and KBM7 cells after exposure to CDT purified from *E. coli* (right). Exposure of cells to CDT results in an increase of cells in the G_2/M phase of the cell cycle (arrow A) and cell death (arrow B). **(B)** Schematic outline of the insertion sites (red lines) in mutant cells unresponsive to CDT. **(C)** Resistance of TMEM181 mutant cells and SGMS1 mutant cells to CDT. Mutant cells reconstituted with the respective cDNAs re-acquire toxin sensitivity. Results are presented as mean values ± SD (error bars) (n = 3 biological replicates). **(D)** Flag-tagged CDT was bound to immobilized anti-Flag antibody and incubated with KBM7 cell lysates or lysates of cells expressing HA-TMEM181. Bound proteins were eluted and subjected to immunoblot analysis. Anti-Flag beads without bound CDT served as a control. **(E)** NIH3T3, U2OS, and HELA cells infected with a TMEM181-expressing retrovirus were treated with increasing amounts of CDT. After 5 days, adherent cells were stained with crystal violet.

stained for influenza nucleoprotein 12 hours after infection. As expected, KBM7 cells showed high levels of infection (~95% infection), whereas CMAS and SLC35A2 mutant cells showed near-complete resistance to virus infection (<0.01% infection) (Fig. 3B and fig. S5D). Absence of CMAS and SLC35A2 expression in the mutants was verified by reverse transcription polymerase chain reaction (RT-PCR) or immunoblot analysis (fig. S5, B and C). Transduction with cDNAs encoding the disrupted genes fully restored susceptibility to influenza infection (Fig. 3B), indicating that the observed resistance is attributable to the mutated loci. Although the KBM7 genome has not been screened at saturation for resistance to influenza, the transporter (SLC35A2) and enzyme (CMAS) identified here could lead to the development of antiviral therapies for influenza.

Identification of host factors for ADP-ribosylating toxins. Diphtheria and anthrax toxins are AB toxins composed of a cell-binding moiety (B) and an active (A) subunit that targets a host function to increase virulence. We have a detailed molecular understanding of how diphtheria toxin enters the cell and induces cell death (*19*, *20*). Can haploid genetic screens identify previously unidentified components in this well-characterized host/pathogen interaction? We screened mutagenized cells with diphtheria or anthrax toxin. Because native anthrax toxin is not cytotoxic for KBM7 cells, we exposed cells to the cell-binding component of anthrax toxin–protective antigen (PA) and anthrax lethal factor (LFN) fused to the catalytic domain of diphtheria toxin (LFN-DTA) (*21*). Resistant mutants were classified as either being resistant to anthrax toxin (PA-LFN-DTA) (class I), resistant to diphtheria toxin (class II), or resistant to both (class III). Mutants in the known anthrax toxin receptor (ANTXR2) (*22*) were recovered with 10 independent insertions and with 12 insertions for the known diphtheria toxin receptor [heparin-binding EGF-like growth factor (HB-EGF)] (Fig. 4A and fig. S6A) (*23*). The third class of mutants included genes involved in diphthamide biosynthesis [DPH1, DPH2, and DPH5; see (*20*)] and a previously uncharacterized gene named WDR85 (Fig. 4A and fig. 6A). All of these insertions were in the same transcriptional orientation as the mutated gene and were thus predicted to impair gene function. In the WDR85 mutant (hereafter referred to as WDR85^{GT}), no WDR85 transcripts were observed, as determined by RT-PCR (Fig. 4B). The resistance of WDR85^{GT} was readily complemented by transduction with WDR85 cDNA, which restored the sensitivity of WDR85^{GT} cells to diphtheria toxin and anthrax toxin (PA-LFN-DTA) (Fig. 4C and fig. S6B). WDR85^{GT} cells were also resistant to *Pseudomonas* exotoxin A, another adenosine diphosphate (ADP)–ribosylating toxin with a similar mode of action as diphtheria toxin (Fig. 4D). Although native anthrax toxin is not lethal to most cell types, including KBM7, its cellular entry and activity can be probed by monitoring cleavage of its cellular target MEK-3. WDR85^{GT} cells were still responsive to the native anthrax toxin, because the extent of proteolytic cleavage of MEK-3 was comparable for WDR85^{GT} and WT cells (fig. S7A), suggesting that toxin entry was normal in WDR85^{GT} cells.

WDR85 is part of the dipthamide biosynthetic pathway. Given the resistance of WDR85 mutant cells to different bacterial toxins, we further explored the mechanism by which WDR85 conferred sensitivity to toxin-mediated cell death. Diphtheria toxin, LFN-DTA, and exotoxin A inhibit host translation through ADP ribosylation of elongation factor 2 (EF2) and thus cause cell death (*19*). ADP ribosylation occurs on diphthamide, a posttranslationally modified histidine uniquely present in EF2 and conserved among all eukaryotes. As WDR85 was not required for toxin entry, we investigated EF2 ribosylation in response to diphtheria toxin. In cell lysates derived from WDR85^{GT} cells, EF2 ADP ribosylation was impaired and could be restored by re-expression of a WDR85 cDNA (fig. S7B). EF2 fused to a streptavidin-binding peptide (SBP) purified from WDR85^{GT} cells was also a poor substrate for ADP ribosylation in vitro (Fig. 5A). Impaired ADP ribosylation is thus an inherent property of EF2 derived from WDR85^{GT} cells and is not due to the presence or absence of other factors present in cell lysates. Diphthamide biosynthesis is the result of stepwise posttranslational modification of His^{175} (fig. S7D), the proteins responsible for which are known (*20*, *24*, *25*). The second step involves the trimethylation of "intermediate" EF2 by the methyltransferase DPH5, with *S*-adenosylmethionine as the methyl donor (*26*). To investigate if this methylation step was affected by the loss of WDR85, we purified intermediate EF2 from DPH5 null cells and performed in vitro methylation assays in cell lysates. Efficient methylation of intermediate EF2 by WT and WDR85^{GT} cell lysates suggested that WDR85 is not required for the second step of diphthamide biosynthesis (Fig. 5B). Next, we purified EF2 from WDR85^{GT} cells and used LC/MS/MS (liquid chromatography–tandem mass spectrometry) to monitor the relevant modifications of His^{175}. Modifications of His^{175} predict an increase in mass by +143 (diphthamide), +142 (diphthine), and +101 (the intermediate) mass units for those peptides that carry the modified His residue. SBP-tagged EF2 isolated from WDR85^{GT} showed a mass consistent with the presence of unmodified His^{175} (Fig. 5C), whereas modifications of EF2 purified from WT and DPH5 mutant cells showed a mass that was expected for the presence of diphthamide and intermediate, respectively (fig. S8, A and B). The absence of modified histidine in EF2 suggests that WDR85 plays a role in the first step in diphthamide biosynthesis.

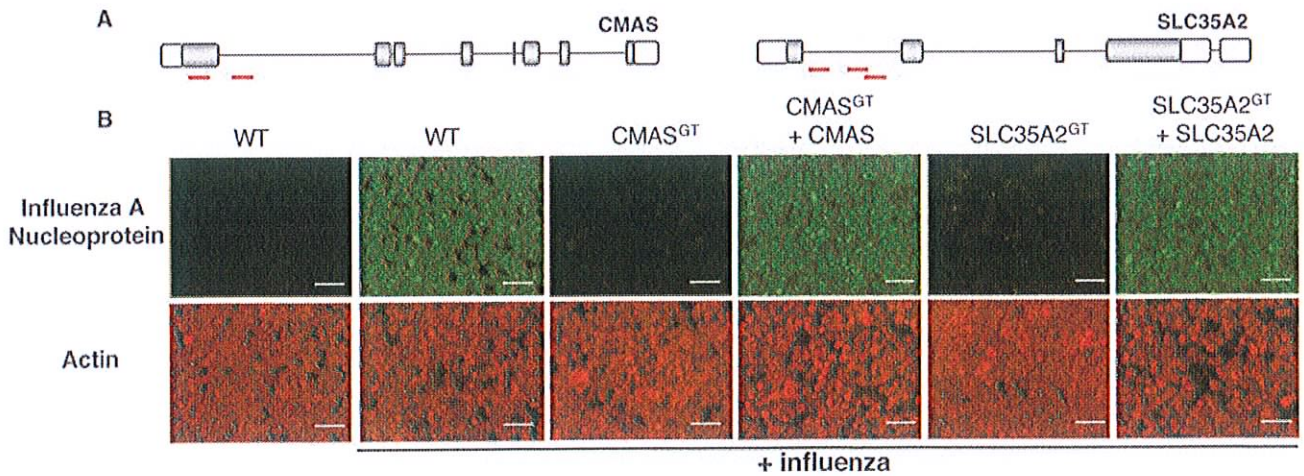

Fig. 3. Cellular genes required for influenza infection. **(A)** Schematic outline of the identified insertion sites (red lines) in the mutated genes. **(B)** Cells were exposed to influenza virus and stained 12 hours later with antibodies directed against influenza A nucleoprotein. Mutant cells reconstituted with cDNAs that correspond to the mutated gene products re-acquire virus sensitivity. Scale bars, 50 μm.

In the course of purification of EF2 from WDR85^{GT} cells, we detected a protein that strongly interacted with EF2 (Fig. 5D). Mass spectrometry and immunoblot analysis identified this protein as DPH5 (Fig. 5E and fig. S7C). WDR85 lacks homology to known proteins involved in diphthamide biosynthesis but does contain WD40 repeats, often involved in protein/protein interactions. Thus, WDR85 may serve as a scaffold to coordinate the association (or dissociation) of enzymatic complexes required for the stepwise biosynthesis of diphthamide.

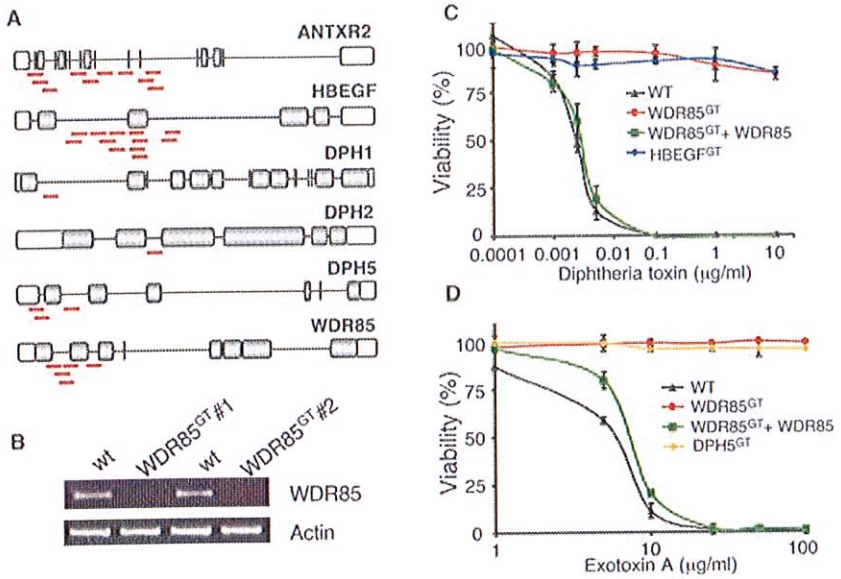

Fig. 4. Identification of loci that confer resistance to ADP-ribosylating bacterial toxins. **(A)** Schematic outline of the insertion sites (red lines) in the mutated genes. **(B)** RT-PCR for WDR85 shows undetectable WDR85 mRNA levels in independent clones with gene-trap insertions in the WDR85 locus. **(C)** Resistance of WDR85^{GT} cells to diphtheria toxin. Error bars indicate SD. **(D)** Resistance of WDR85^{GT} cells to exotoxin A. Identified clones with mutations in HB-EGF and DPH5 served as insensitive controls for these respective toxins, and WDR85^{GT} cells reconstituted with a WDR85 cDNA (WDR85^{GT} + WDR85) re-acquired sensitivity to the toxins. Results are presented as mean values ± SD (n = 3).

WDR85 is a conserved protein with homology to yeast YBR246W (fig. S9A). We used a database containing fitness profiles of deletion strains of all nonessential yeast genes under 1144 chemical conditions to cluster genes with similar profiles to YBR246W (*27*). The top 10 genes that phenoclustered with YBR246W by homozygous cosensitivity included DPH2 and DPH5 (fig. S9, B and C). The only gene annotation terms we found enriched concerned diphthamide biosynthesis [P value = 9×10^{-4} (fig. S9C)]. To directly test whether YBR246W is involved in diphthamide biosynthesis, we undertook ribosylation assays in protein extracts derived from WT yeast or yeast strains deleted for YKL191W (DPH2) or YBR246W. Deficiency of YKL191W and YBR246W both impaired ADP-ribosyl acceptor activity of EF2 in yeast (Fig. 5F). Thus, the role of WDR85 in diphthamide biosynthesis is conserved in eukaryotes, and the proposed scaffolding role may be the main function of WDR85 in cells. WDR85 therefore represents a host gene involved in the first step in diphthamide biosynthesis, despite previous suggestions that all proteins involved in this complex posttranslational modification were known (*20*).

Perspective. One of the main strengths of yeast as a genetic tool is the relative ease with which recessive mutations can be recognized and characterized at its haploid life stage (*28*), a trait absent from the commonly studied higher eukaryotes. The approach described here will allow similar types of screens for the human genome.

The discovery of RNA interference has enabled targeted reduction of gene expression in diploid cultured mammalian and insect cells, which

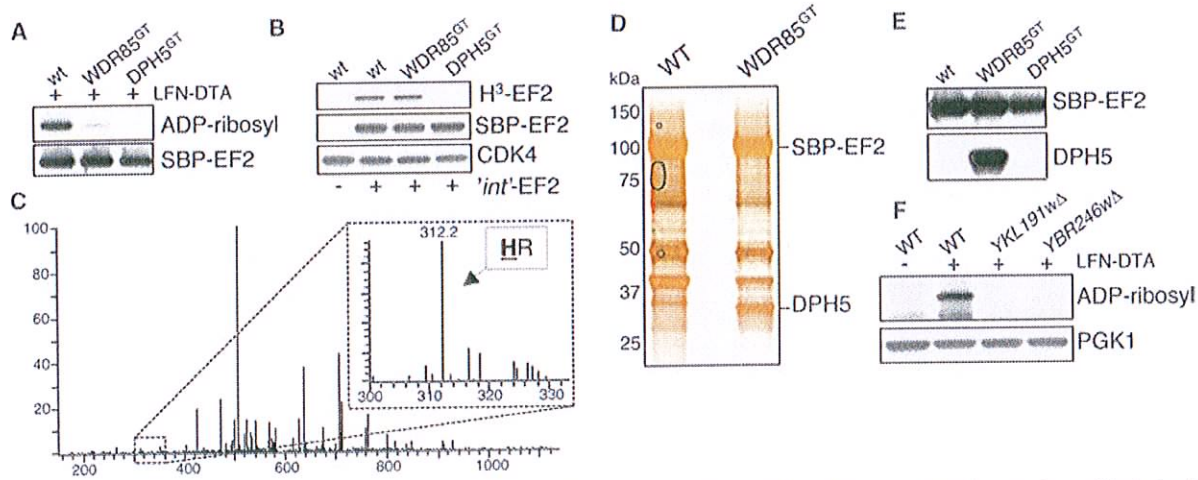

Fig. 5. WDR85 is involved in diphthamide biosynthesis. **(A)** In vitro ADP ribosylation of SBP-tagged EF2 purified from WT, WDR85, and DPH5 mutant cells by LFN-DTA in the presence of nicotinamide adenine dinucleotide (NAD)–biotin. Streptavidin–horseradish peroxidase (HRP) was used to detect ADP ribosylation, and total EF2 was detected by immunoblot analysis. **(B)** Methylation of intermediate EF2 by WT, WDR85, and DPH5 mutant cell lysates. SBP-tagged intermediate EF2 was purified from DPH5 mutant cells and incubated in lysates derived from the indicated genotypes in the presence of [methyl-^{3}H] adenosylmethionine (Ado-S-Me) as methyl donor. The amount of supplied intermediate EF2 was detected by immunoblot analysis, with CDK4 as a loading control. **(C)** MS/MS spectra of a tryptic peptide derived from SBP-tagged EF2 purified from WDR85 mutant cells. Peptide fragments characteristic for unmodified His^{715} are indicated. **(D)** Silver stain of SPB-EF2 purified from WT and WDR85-deficient cells. kDa, kilodaltons. **(E)** Immunoblot analysis of SBP-EF2 pull-down from WT, WDR85-, and DPH5-deficient cells probed with an antibody directed against DPH5. **(F)** Protein extracts from WT, YKL191W-, and YBR246W-deficient *S. cerevisiae* strains were incubated with LFN-DTA in the presence of NAD-biotin. Streptavidin-HRP was used to detect ADP ribosylation, and PGK1 was used as loading control.

opened the door to large-scale screens. At the same time, limitations of this approach are increasingly apparent, such as the induction of off-target effects that complicate genome-wide screens in particular (*29*, *30*) and the inability to completely switch off gene expression. When similar small interfering RNA screens are conducted independently in mammalian cells, the lack of concordance between them is an additional complicating factor (*31*, *32*). Finally, mammals are rather robust in their tolerance to partial loss of gene function: Haploinsufficiency appears to be the exception rather than the rule, because inactivation of one gene copy, as in heterozygous knockout mice, rarely leads to severe phenotypes.

Although we have focused on host-pathogen biology, similar screens could in principle be applied to any phenotype that can be recognized in a population of mutant cells, such as modulation of a genetically encoded reporter. In the future, haploid genetic screens could be used to generate comprehensive compendia of host factors that are used by different pathogens and may yield new strategies to combat infectious disease. In conclusion, the haploid genetic screens described here expand mutagenesis-based screens in model organisms by providing a window on disease-associated molecular networks that can be studied in cultured human cells.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/326/5957/1231/DC1
Materials and Methods
Figs. S1 to S9
References

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Proteome Organization in a Genome-Reduced Bacterium

Sebastian Kühner,[1]* Vera van Noort,[1]* Matthew J. Betts,[1] Alejandra Leo-Macias,[1] Claire Batisse,[1] Michaela Rode,[1] Takuji Yamada,[1] Tobias Maier,[2] Samuel Bader,[1] Pedro Beltran-Alvarez,[1] Daniel Castaño-Diez,[1] Wei-Hua Chen,[1] Damien Devos,[1] Marc Güell,[2] Tomas Norambuena,[3] Ines Racke,[1] Vladimir Rybin,[1] Alexander Schmidt,[4] Eva Yus,[2] Ruedi Aebersold,[4] Richard Herrmann,[5] Bettina Böttcher,[1]† Achilleas S. Frangakis,[1] Robert B. Russell,[1] Luis Serrano,[2,6] Peer Bork,[1]‡ Anne-Claude Gavin[1]‡

The genome of *Mycoplasma pneumoniae* is among the smallest found in self-replicating organisms. To study the basic principles of bacterial proteome organization, we used tandem affinity purification–mass spectrometry (TAP-MS) in a proteome-wide screen. The analysis revealed 62 homomultimeric and 116 heteromultimeric soluble protein complexes, of which the majority are novel. About a third of the heteromultimeric complexes show higher levels of proteome organization, including assembly into larger, multiprotein complex entities, suggesting sequential steps in biological processes, and extensive sharing of components, implying protein multifunctionality. Incorporation of structural models for 484 proteins, single-particle electron microscopy, and cellular electron tomograms provided supporting structural details for this proteome organization. The data set provides a blueprint of the minimal cellular machinery required for life.

Biological function arises in part from the concerted actions of interacting proteins that assemble into protein complexes and networks. Protein complexes are the first level of cellular proteome organization: functional and structural units, often termed molecular machines, that participate in all major cellular processes. Complexes are also highly dynamic in the sense that their organization and composition vary in time and space (*1*), and they interact to form higher level networks; this property is central to whole-cell functioning. However, general rules concerning protein complex assembly and dynamics remain elusive.

The combination of affinity purification with mass spectrometry (MS) (*2*) has been applied to several organisms to provide a growing repertoire of molecular machines. Genome-wide screens in *Saccharomyces cerevisiae* (*3*–*5*) captured discrete, dynamic proteome organization and revealed higher-order assemblies with direct connections between complexes and frequent sharing of common components. To date these exhaustive analyses have been applied only in yeast. In bacteria, genome-wide yeast two-hybrid analyses have been reported (*6*, *7*), but only a few biochemical analyses on selected sets of complexes are available (*8*–*11*). The understanding of proteome organization in these organisms concerns thus the binary interaction networks.

Here, we report a genome-scale analysis of protein complexes in the bacterium *Mycoplasma pneumoniae*, a human pathogen that causes atypical

[1]European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117 Heidelberg, Germany. [2]Centro Regulacion Genomica–Universidad Pompeu Fabra, Dr Aiguader 88, 08003 Barcelona, Spain. [3]Pontificia Universidad Catolica de Chile, Alameda 340, Santiago, Chile. [4]ETH (Eidgenössische Technische Hochschule) Zürich, Wolfgang-Pauli-Strasse 16, 8093 Zürich, Switzerland; Faculty of Science, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland, and Institute for Systems Biology, Seattle, WA 98013, USA. [5]ZMBH (Zentrum für Molekulare Biologie der Universität Heidelberg), Im Neuenheimer Feld 282, 69120 Heidelberg, Germany. [6]ICREA (Institució Catalana de Recerca i Estudis Avançats), 08010 Barcelona, Spain.

*These authors contributed equally to this work.
†Present address: University of Edinburgh, Kings Buildings, Mayfield Road, Edinburgh EH9 3JR.
‡To whom correspondence should be addressed. E-mail: gavin@embl.de (A.-C.G.); bork@embl.de (P.B.)

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Approved GMO	pTRK830, pOR128, pTRK669	
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We found that cathepsin B is involved in autophagy flux and defects in the flux causes cell death. NPC-1 deficeint cells are defective in autophagy flux. We will examine if this cell types are more sensitive to autophagy induced cell death. Other cell types are for control or to examine cell death induced by LPS or anthrax toxins.
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Receptors:	vitronectin, expressed
Tumorigenic:	Yes
DNA Profile (STR):	Amelogenin: X CSF1PO: 11,12 D13S317: 12,14 D16S539: 9,13 D5S818: 8,9 D7S820: 11,12 THO1: 7,9.3 TPOX: 11 vWA: 16,19
Cytogenetic Analysis:	

Related Links ▸

- NCBI Entrez Search
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This is a hypotriploid human cell line. The modal chromosome number was 64, occurring in 30% of cells. The rate of cells with higher ploidies was 4.2 %. The der(1)t(1;15) (q42;q13), der(19)t(3;19) (q12;q13), der(12)t(8;12) (q22;p13), and four other marker chromosomes were common to most cells. Five other markers occurred in some cells only. The marker der(1) and M8 (or Xq+) were often paired. There were four copies of N17 and N22. Noticeably in addition to three copies of X chromosomes, there were paired Xq+, and a single Xp+ in most cells.

Age: fetus

Comments:

Although an earlier report suggested that the cells contained Adenovirus 5 DNA from both the right and left ends of the viral genome [RF32764], it is now clear that only left end sequences are present. [39768]
The line is excellent for titrating human adenoviruses.
The cells express an unusual cell surface receptor for vitronectin composed of the integrin beta-1 subunit and the vitronectin receptor alpha-v subunit. [23406]
The Ad5 insert was cloned and sequenced, and it was determined that a colinear seqment from nts 1 to 4344 is integrated into chromosome 19 (19q13.2). [39768]

Propagation:

ATCC complete growth medium: The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.
Atmosphere: air, 95%; carbon dioxide (CO2), 5%
Temperature: 37.0°C
The cell line does not adhere to the substrate when left at room temperature for any length of time, therefore, live cultures may be received with the cells detached.The cells will re-attach to the flask over a period of several days in culture at 37C.

Subculturing:

Cell Biology

ATCC® Number:	**HB-8065™**	Order this Item	Price:	**$264.00**

Designations:	Hep G2
Depositors:	Wistar Institute
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Homo sapiens* (human)
Morphology:	epithelial 
Source:	**Organ:** liver **Disease:** hepatocellular carcinoma
Cellular Products:	alpha-fetoprotein (alpha fetoprotein); albumin; alpha2 macroglobulin (alpha-2-macroglobulin); alpha1 antitrypsin (alpha-1-antitrypsin); transferrin; alpha1 antichymotrypsin; (alpha-1-antichymotrypsin); haptoglobin; ceruloplasmin; plasminogen; [3525] complement (C4); C3 activator; fibrinogen; alpha1 acid glycoprotein (alpha-1 acid glycoprotein); alpha2 HS glycoprotein (alpha-2-HS-glycoprotein); beta lipoprotein (beta-lipoprotein); retinol binding protein (retinol-binding protein) [3525]
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Applications:	transfection host (Nucleofection technology from Lonza Roche FuGENE® Transfection Reagents)
Receptors:	insulin; insulin-like growth factor II (IGF II) [22446]
Tumorigenic:	No

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DNA Profile (STR):

	Amelogenin: X,Y CSF1PO: 10,11 D13S317: 9,13 D16S539: 12,13 D5S818: 11,12 D7S820: 10 F13A01: 5,7 F13B: 6,10 FESFPS: 11 LPL: 10,11 THO1: 9 TPOX: 8,9 vWA: 17
Cytogenetic Analysis:	modal number = 55 (range = 50 to 60); has a rearranged chromosome 1 [3525]
Age:	15 years adolescent
Gender:	male
Ethnicity:	Caucasian
Comments:	The cells express 3-hydroxy-3-methylglutaryl-CoA reductase and hepatic triglyceride lipase activities. [23557] The cells demonstrate decreased expression of apoA-I mRNA and increased expression of catalase mRNA in response to gramoxone (oxidative stress). [26594] There is no evidence of a Hepatitis B virus genome in this cell line. [1205] [22909]
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Temperature:** 37.0°C

Subculturing:

Cell Biology

ATCC® Number:	**CCL-61™**	Order this Item	Price:	**$256.00**

Designations:	CHO-K1
Depositors:	TT Puck
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	Cricetulus griseus (hamster, Chinese)
Morphology:	epithelial-like 
Source:	**Organ:** ovary
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Isolation:	**Isolation date:** 1957
Applications:	transfection host (Nucleofection technology from Lonza Roche FuGENE® Transfection Reagents)
Virus Susceptibility:	vesicular stomatitis (Indiana); Getah virus
Virus Resistance:	poliovirus 2; modoc virus; Button Willow virus
Reverse Transcript:	negative
Cytogenetic Analysis:	Chromosome Frequency Distribution 50 Cells: 2n = 22. Stemline number is hypodiploid.
Gender:	female
Comments:	The CHO-K1 cell line was derived as a subclone from the parental CHO cell line initiated from a biopsy of an ovary of an adult Chinese hamster by T. T. Puck in 1957. [22224] The cells require proline in the medium for growth. [25976]
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated F-12K Medium, Catalog No. 30-2004. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Temperature:** 37.0°C

Related Links ▸
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Related Cell Culture Products

Protocol:

Subculturing:

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53 mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:4 to 1:8 is recommended
Medium Renewal: Once or twice between subculture

Preservation:
Freeze medium: Complete growth medium 95%; DMSO, 5%
Storage temperature: liquid nitrogen vapor phase
recommended serum:ATCC 30-2020

Related Products: Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2004

Cell Biology

ATCC® Number:	HTB-37™	Order this Item	Price: $264.00

Designations:	Caco-2
Depositors:	J Fogh
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Homo sapiens* (human)
Morphology:	epithelial 
Source:	**Organ:** colon **Disease:** colorectal adenocarcinoma
Cellular Products:	keratin retinoic acid binding protein I retinol binding protein 2
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Restrictions:	The cells are distributed for research purposes only. The Memorial Sloan-Kettering Cancer Center releases the line subject to the following: 1.) The cells or their products must not be distributed to third parties. Commercial interests are the exclusive property of Memorial Sloan-Kettering Cancer Center. 2.) Any proposed commercial use of these cells must first be negotiated with The Director, Office of Industrial Affairs, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021; phone (212) 639-6181; FAX (212) 717-3439.
Applications:	transfection host (Nucleofection technology from Lonza Roche FuGENE® Transfection Reagents)
Receptors:	heat stable enterotoxin (Sta, E. coli), expressed epidermal growth factor (EGF), expressed
Virus Susceptibility:	Human immunodeficiency virus 1
Tumorigenic:	Yes
Reverse Transcript:	N

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Cytogenetic Analysis:	The stemline modal chromosome number is 96, occurring at 16% with polyploidy at 3.2%. Ten common markers were detected i.e., t(1q;?), 10q-, t(11q17q) and 7 others. The t (1q17q) and M11 were found in a portion of cells. The ins(2), 10q-, and t(15q;?) were generally paired, and t(11q;17q) and t (21q;?) were mostly three-copied. Normal N9 was absent, and N21 was lost in some cells. One to 4 small acrocentric chromosomes were detected. No Y chromosome with bright distal q-band was detected by Q-observation.
Isoenzymes:	AK-1, 1 ES-D, 1 G6PD, B GLO-I, 1 Me-2, 1 PGM1, 1 PGM3, 1
Age:	72 years adult
Gender:	male
Ethnicity:	Caucasian
HeLa Markers:	N
Comments:	Upon reaching confluence, the cells express characteristics of enterocytic differentiation [PubMed ID: 1939345]. Caco-2 cells express retinoic acid binding protein I and retinol binding protein II [PubMed ID: 9040537].
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 20%. **Atmosphere:** air, 95%; carbon dioxide (CO2), 5% **Temperature:** 37.0°C
Subculturing:	

Catalog ID: **GM00038**

Product (Source): CELL CULTURE

ADD TO CART

Overview

Collection NIGMS Human Genetic Cell Repository
Subcollection Apparently Healthy Collection
Sample Description APPARENTLY HEALTHY NON-FETAL TISSUE
Biopsy Source Unspecified
Cell Type Fibroblast
Tissue Type Skin
Transformant Untransformed
Species Homo sapiens
Common Name Human
Age 9 YR
Sex Female
Race Black
Family 163 View Pedigree
Family Member 1
Relation to Proband daughter
Clinically Affected No
Confirmation Karyotypic analysis and Case history
Remarks 46,XX; 2% of cells show random chromosome loss and 4% show random chromosomal aberrations; skin biopsy; mother is GM00043B

Catalog ID GM00038
Product Cell Culture
Pricing Commercial Pricing: $85.00
Academic and not-for-profit pricing: $85.00
How to Order Online Ordering
Assurance Form (Must have current form on file)
Statement of Research Intent Form (Information will be entered electronically when order is placed. DO NOT fax form to Coriell Customer Service)

Characterizations

Sample Description APPARENTLY HEALTHY NON-FETAL TISSUE
Passage Frozen 10

IDENTIFICATION OF SPECIES OF ORIGIN Species of Origin Confirmed by Nucleoside Phosphorylase, Glucose-6-Phosphate Dehydrogenase, and Lactate Dehydrogenase Isoenzyme Electrophoresis and by Chromosome Analysis

Phenotypic Data

Remark 46,XX; 2% of cells show random chromosome loss and 4% show random chromosomal aberrations; skin biopsy; mother is GM00043B

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Tan YH, Greene AE, Subregional localization of the gene(s) governing the human interferon induced antiviral state in man. J Gen Virol32:153-5 1976
PubMed ID: 182912

Tan YH, Chou EL, Lundh N, Regulation of chromosome 21-directed anti-viral gene(s) as a consequence of age. Nature257:310-2 1975
PubMed ID: 1161032

External Links

dbSNP dbSNP ID: 21465
GEO GEO Accession No: GSM88289
GEO Accession No: GSM88290
GEO Accession No: GSM88291
GEO Accession No: GSM88307
GEO Accession No: GSM88308
GEO Accession No: GSM88309

Images

View pedigree

Protocols

Passage Frozen 10
Split Ratio 1:4
Temperature 37 C
Percent CO2 5%
Medium Eagle's Minimum Essential Medium with Earle's salts and non-essential amino acids
Serum 15% fetal bovine serum Not inactivated

Catalog ID: **GM03123** Product (Source): CELL CULTURE ADD TO CART

Overview

Collection NIGMS Human Genetic Cell Repository
Subcollection Inherited Disorders
Class Disorders of Lipid Metabolism
Sample Description NIEMANN-PICK DISEASE, TYPE C1; NPC1
NPC1 GENE; NPC1
Cell Type Fibroblast
Transformant Untransformed
Species Homo sapiens
Common Name Human
Age 9 YR
Sex Female
Race Caucasian
Family 451
Family Member 1
Relation to Proband proband
Clinically Affected Yes
Confirmation Biochemical characterization after cell line submission to CCR
Remarks See GM03124 Lymphoid; 38% of normal sphingomyelinase activity, normal B-galactosidase activity, and impaired cholesterol esterification in fibroblasts; the donor subject is a compound heterozygote; one allele carries a missense mutation C>T at nucleotide 709 (709C>T) in exon 6 of the NPC1 gene, resulting in a substitution of a serine for a proline at codon 237 [Pro237Ser (P237S)]; the second allele also carries a missense mutation T>C at nucleotide 3182 (3182T>C) in exon 21 which results in the substitution of a threonine for an isoluecine at codon 1061 [Ile1061Thr (I1061T)] in a transmembrane domain.

Catalog ID GM03123
Product Cell Culture
Pricing Commercial Pricing: $85.00
Academic and not-for-profit pricing: $85.00
How to Order Online Ordering
Assurance Form (Must have current form on file)
Statement of Research Intent Form (Information will be entered electronically when order is placed. DO NOT fax form to Coriell Customer Service)

Characterizations

Sample Description NIEMANN-PICK DISEASE, TYPE C1; NPC1
NPC1 GENE; NPC1
Passage Frozen 5

IDENTIFICATION OF SPECIES OF ORIGIN Species of Origin Confirmed by Nucleoside Phosphorylase, Glucose-6-Phosphate Dehydrogenase, and Lactate Dehydrogenase Isoenzyme Electrophoresis

sphingomyelin phosphodiesterase According to the submitter, biochemical test results for this subject showed decreased enzyme activity. EC Number: 3.1.4.12; 38% activity.

Gene NPC1
Chromosomal Location 18q11-q12
Allelic Variant 1 P237S; NIEMANN-PICK DISEASE, TYPE C1
Identified Mutation **PRO237SER**

Gene NPC1
Chromosomal Location 18q11-q12
Allelic Variant 2 607623.0010; NIEMANN-PICK DISEASE, TYPE C1
Identified Mutation **ILE1061THR**; In an initial study of 25 patients with type C1 Niemann-Pick disease, Millat et al. [Am. J. Hum. Genet. 65: 1321-1329 (1999)] identified a T-to-C transition at nucleotide 3182 of the NPC1 gene that led to an Ile1061-to-Thr substitution (I1061T) in 3 patients. The mutation, located in exon 21, affected a putative transmembrane domain of the protein. The mutation was particularly frequent in patients with NPC from western Europe, especially France and the U.K. and in Hispanic patients whose roots were in the Upper Rio Grande valley of the U.S. Millat et al. [Am. J. Hum. Genet. 65: 1321-1329 (1999)] concluded that the I1061T mutation originated in Europe and that the high frequency in northern Rio Grande Hispanics resulted from a founder effect.

Phenotypic Data

Remark See GM03124 Lymphoid; 38% of normal sphingomyelinase activity, normal B-galactosidase activity, and impaired cholesterol esterification in fibroblasts; the donor subject is a compound heterozygote; one allele carries a missense mutation C>T at nucleotide 709 (709C>T) in exon 6 of the NPC1 gene, resulting in a substitution of a serine for a proline at codon 237 [Pro237Ser (P237S)]; the second allele also carries a missense mutation T>C at nucleotide 3182 (3182T>C) in exon 21 which results in the substitution of a threonine for an isoluecine at codon 1061 [Ile1061Thr (I1061T)] in a transmembrane domain.

Publications

Ahras M, Naing T, McPherson R, Scavenger receptor class B type I localizes to a late endosomal compartment Journal of lipid research49:1569-76 2008
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Yamauchi Y, Reid PC, Sperry JB, Furukawa K, Takeya M, Chang CC, Chang TY, Plasma membrane rafts complete cholesterol synthesis by participating in retrograde movement of precursor sterols The Journal of biological chemistry282:34994-5004 2007
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Ganley IG, Pfeffer SR, Cholesterol accumulation sequesters Rab9 and disrupts late endosome function in NPC1-deficient cells The Journal of biological chemistry281:17890-9 2006
PubMed ID: 16644737

Linder MD, Uronen RL, Hölttä-Vuori M, van der Sluijs P, Peränen J, Ikonen E, Rab8-dependent recycling promotes endosomal cholesterol removal in normal and sphingolipidosis cells Molecular biology of the cell18:47-56 2006
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Narita K, Choudhury A, Dobrenis K, Sharma DK, Holicky EL, Marks DL, Walkley SU, Pagano RE, Protein transduction of Rab9 in Niemann-Pick C cells reduces cholesterol storage The FASEB journal : official publication of the Federation of American Societies for Experimental Biology19:1558-60 2005
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Chen FW, Gordon RE, Ioannou YA, NPC1 late endosomes contain elevated levels of non-esterified ('free') fatty acids and an abnormally glycosylated form of the NPC2 protein The Biochemical journal390:549-61 2005
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Scott C, Higgins ME, Davies JP, Ioannou YA, Targeting of NPC1 to late endosomes involves multiple signals, including one residing within the putative sterol-sensing domain The Journal of biological chemistry279:48214-23 2004
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White NM, Corey DA, Kelley TJ, Mechanistic similarities between cultured cell models of cystic fibrosis and Niemann-Pick type C. Am J Respir Cell Mol BiolEpub ahead of print:48214-23 2004
PubMed ID: 15256387

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PubMed ID: 15292453

Choi HY, Karten B, Chan T, Vance JE, Greer WL, Heidenreich RA, Garver WS, Francis GA, Impaired ABCA1-dependent lipid efflux and hypoalphalipoproteinemia in human Niemann-Pick type C disease. J Biol Chem278(35):32569-77 2003
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PubMed ID: 12867541

Walter M, Davies JP, Ioannou YA, Telomerase immortalization upregulates Rab9 expression and restores LDL cholesterol egress from Niemann-Pick C1 late endosomes. J Lipid Res44(2):243-53 2003
PubMed ID: 12576506

Choudhury A, Dominguez M, Puri V, Sharma DK, Narita K, Wheatley CL, Marks DL, Pagano RE, Rab proteins mediate Golgi transport of caveola-internalized glycosphingolipids and correct lipid trafficking in Niemann-Pick C cells. J Clin Invest109(12):1541-50 2002
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Sun X, Marks DL, Park WD, Wheatley CL, Puri V, O'Brien JF, Kraft DL, Lundquist PA, Patterson MC, Pagano RE, Snow K, Niemann-Pick C variant detection by altered sphingolipid trafficking and correlation with mutations within a specific domain of NPC1. Am J Hum Genet68(6):1361-72 2001
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Kruth HS, Ifrim I, Chang J, Addadi L, Perl-Treves D, Zhang WY, Monoclonal antibody detection of plasma membrane cholesterol microdomains responsive to cholesterol trafficking. J Lipid Res42(9):1492-500 2001
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Yamamoto T, Ninomiya H, Matsumoto M, Ohta Y, Nanba E, Tsutsumi Y, Yamakawa K, Millat G, Vanier MT, Pentchev PG, Ohno K, Genotype-phenotype relationship of Niemann-Pick disease type C: a possible correlation between clinical onset and levels of NPC1 protein in isolated skin fibroblasts. J Med Genet37(9):707-12 2000
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Gu JZ, Carstea ED, Cummings C, Morris JA, Loftus SK, Zhang D, Coleman KG, Cooney AM, Comly ME, Fandino L, Roff C, Taglo DA, Pavan WJ, Pentchev PG, Rosenfeld MA, Substantial narrowing of the Niemann-Pick C candidate interval by yeast artificial chromosome complementation. Proc Natl Acad Sci U S A94:7378-83 1997
PubMed ID: 9207099

Liscum L, Faust JR, The intracellular transport of low density lipoprotein-derived cholesterol is inhibited in Chinese hamster ovary cells cultured with 3-beta-[2-(diethylamino)ethoxy]androst-5-en-17-one. J Biol Chem264(20):11796-806 1989
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Pentchev PG, Comly ME, Kruth HS, Vanier MT, Wenger DA, Patel S, Brady RO, A defect in cholesterol esterification in Niemann-Pick disease (type C) patients. Proc Natl Acad Sci U S A82:8247-51 1985
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Maziere JC, Maziere C, Mora L, Routier JD, Polonovski J, In situ degradation of sphingomyelin by cultured normal fibroblasts and fibroblasts from patients with Niemann-Pick disease type A and C. Biochem Biophys Res Commun108:1101-6 1982
PubMed ID: 7181884

External Links

dbSNP	dbSNP ID: 17690
Gene Cards	NPC1
Gene Ontology	GO:0004888 transmembrane receptor activity
	GO:0005478 intracellular transporter activity
	GO:0005624 membrane fraction
	GO:0005764 lysosome
	GO:0006886 intracellular protein transport
	GO:0008158 hedgehog receptor activity
	GO:0015248 sterol transporter activity
	GO:0016021 integral to membrane
	GO:0030301 cholesterol transport
Locus Link	LocusLink ID: 4864
OMIM	257220 NIEMANN-PICK DISEASE, TYPE C1; NPC1
	607623 NPC1 GENE; NPC1
Omim Description	NIEMANN-PICK DISEASE WITH CHOLESTEROL ESTERIFICATION BLOCK
	NIEMANN-PICK DISEASE, CHRONIC NEURONOPATHIC FORM
	NIEMANN-PICK DISEASE, SUBACUTE JUVENILE FORM
	NIEMANN-PICK DISEASE, TYPE C; NPC
	NIEMANN-PICK DISEASE, TYPE C1; NPC1

Images

Data are not available

Protocols

Passage Frozen	5
Split Ratio	1:5
Temperature	37 C
Percent CO2	5%
Medium	Eagle's Minimum Essential Medium with Earle's salts and non-essential amino acids
Serum	10% fetal bovine serum Not inactivated
Substrate	None specified
Subcultivation Method	trypsin-EDTA

Modification Form for Permit BIO-UWO-0147

Permit Holder: Sung Kim

Approved Personnel
(Please stroke out any personnel to be removed)

Boram Ham

Anthony Bruni

Sarah Spanton

Andrew Martins

Soon-Duck Ha

Additional Personnel
(Please list additional personnel here)

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. *
Approved Microorganisms	P. aeurogenosa, S. typhimurium	Lactobacillus rhamnosus, E.coli EC1000, E.coli BL21
Approved Cells	Human (primary), rodent (primary), human (established), THP-1, rodent (established), RAW 2649	
Approved Use of Human Source Material	Blood (whole), PARF-CFP, mRFP-Rab7, mRFP-Rab5	
Approved GMO		pTRK830, pOR128, pTRK669. "
Approved use of Animals	mice	

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED.*

Classification: 2

Date of last Biohazardous Agents Registry Form: Apr 28, 2008

Signature of Permit Holder

BioSafety Officer(s): N. Hanley Oct 5/09

Chair,Biohazards Subcommittee: M. Kidder

Modification Form for Permit BIO-UWO-0147

Permit Holder: Sung Kim

Approved Toxin(s) cholera, diphtheria, CONT'D anthrax toxin

A Researcher confirmed that the "Biosecurity Requirements Using Biological Agents" will be followed. See attached email (and requirements)

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED.*

Classification: 2

Date of last Biohazardous Agents Registry Form: Apr 28, 2008

Signature of Permit Holder

BioSafety Officer(s):

Chair,Biohazards Subcommittee: Oct 5/09

Subject: [Fwd: Re: [Fwd: Re: [Fwd: [Fwd: Re: Anthrax toxin project]]]]
From: Sung Kim <Sung.Kim@schulich.uwo.ca>
Date: Fri, 02 Oct 2009 09:03:18 -0400
To: Jennifer Stanley <jstanle2@uwo.ca>

Hi Jennifer,
Yes, we will follow the requirements.
Thanks
Sk

Sung O. Kim Ph.D.
Assistant Professor
Department of Microbiology & Immunology
University of Western Ontario

Siebens Drake Research Institute, Rm119A
1400 Western Road
London, Ontario N6G2V4
e-mail: sung.kim@schulich.uwo.ca
Phone: (519)850 2961
Fax: (519)661-2046
http://publish.uwo.ca/~skim283/

>>> Jennifer Stanley <jstanle2@uwo.ca> 28/09/2009 12:47 pm >>>
Hi Dr. Kim
Please send me an e-mail confirming that you will follow the biosecurity requirements.
Thanks
Jennifer

Biosecurity Requirements for Facilities Using Biological Agents

(1) Biological agents protected by a lock. For example, biological agents in a freezer, fridge, laboratories or other type of container must be locked after-hours/if no one present.

(2) The supervisor must ensure that each person has the qualifications and training to do the work without supervision.

(3) Visitors must be accompanied.

(4) The supervisor must keep a current inventory and a list of the location(s) where the biological agent(s) are stored and handled.

(5) Labelling to identify samples and the container in which they are stored.

(6) Notify the biosafety officer if a sample is lost, stolen, or otherwise misused.

(7) Notify Campus Community Police Services of suspicious behaviour.

There are two additional requirements for Facilities Using or Storing Biological Toxins:

(8) Do not keep on hand more than the amounts regulated by the United States Select Agents regulation: www.selectagents.gov/index.htm/

(9) For best practices, it is recommended to use or handle less than one human dose at any given time.

Subject: Re: Use of lethal toxin
From: Sung Kim <Sung.Kim@schulich.uwo.ca>
Date: Fri, 06 Feb 2009 11:22:01 -0500
To: Jennifer Stanley <jstanle2@uwo.ca>

See below:

>>> Jennifer Stanley <jstanle2@uwo.ca> 2/6/2009 11:18 AM >>>
Thanks Dr. Kim

I understand from this that you store ~ 1 mg of each component. Total we have. We have in aliquots.
Do you have any LD50 information? about 100 micg/20g wt. in mice.
How do you dispose of the material when the experiments are complete? Toxin is inactivated in 3h at room temp. Bleach them, anyway.

Jennifer

Sung Kim wrote:
> Hi Jennifer:
>
> >>> Jennifer Stanley <jstanle2@uwo.ca> 2/5/2009 9:36 AM >>>
> Hi Dr. Kim
> Just a couple more questions:
> - how much of the toxins (PA and LF) do you keep on hand at once? ~1
> mg each
> - how much of each do you usually use at once? 5 micg What is the
> concentration of toxin? 50 micg/ml
> Thanks!
> Jennifer

The MTA is to obtain vectors to transform Lactobacillus rhamnosus for probiotic study. We will transform L. rhamnosus using these vectors to identify genes involved in macrophage activation in vitro.

pTRK830 (EmR vector for Lactobacillus rhamnosus) -
pORI28 (integration targeting plasmid, EmR)
pTRK669 (helper plasmid, CmR)
E. coli EC1000
/Purpose is for cloning and integration experiments in Lactobacillus rhamnosus.

We prepare recombinant lethal toxin and protective antigen from E. coli. After purification, we use them cell lines or primary peritoneal or bone marrow-derived macrophages in vitro. All toxins are kept in -80 in our lab. Our lab is locked all the time, unless some one is in site.

We are using E. coli-BL21 to express PA and Bacillus megaterium for LF. I believe they are commercial strains.
Mostly we use both toxins to treat cells.

We use toxins to treat mouse primary and immortalized macrophages, but sometimes use human or mouse fibroblasts.

-------- Original Message --------

Subject: Re: Containment Question, lethal toxin
Date: Fri, 23 Jan 2009 11:10:08 -0500
From: Geneviève Lacroix <genevieve_lacroix@phac-aspc.gc.ca>
To: Jennifer Stanley <jstanle2@uwo.ca>

Good morning Ms. Stanley,

The toxins you are describing me are 2 of the 3 components of anthrax
toxin. There are too many variables for me to give you an answer.
Although the toxins are produced separately in another host, the risk
level is most probably the same, the toxins are probably as potent as
if they were produced by B. anthracis. Once I receive the importation
application, I will have to complete an in depth risk assessment, which
will take some time.

However, I think this information will be useful to you.

Bacillus anthracis causes anthrax. B. anthracis requires 2 plasmids
for its virulence. One plasmid contains the toxin genes (pXO1) and the
second plasmid contains the capsular genes (pXO2). The exotoxins
secreted by B. anthracis, encoded by pXO1, are composed of three
distinct components: protective antigen (PA), lethal factor (LF), and
edema factor (EF). These proteins play a key role in the pathogenesis
of anthrax. EF and LF have enzymatic functions but require PA,
responsible for their transport into the host, to achieve their
biological effects. These proteins individually cause no known
physiological effects in animals but in pairs produce two
toxic actions. Injection of PA with LF causes death of rats in 60 min,
whereas PA with EF causes edema in the skin of rabbits and guinea pigs.
S H Leppla, Anthrax toxin edema factor: a bacterial adenylate cyclase
that increases cyclic AMP concentrations of eukaryotic cells. PNAS May
1, 1982 vol. 79 no. 10 3162-3166.

This is as much as I can do for now. I hope this information will help
you.

Regards

Genevieve Lacroix
A/Head, Importation and Biosafety Program
Chef Intérimaire, Importation et [illegible] de biosécurité
Office of Laboratory Security / Bureau de la sécurité des laboratoires
Public Health Agency of Canada / Agence de la santé publique du Canada
[illegible], Ottawa, Ontario, Canada [illegible]
[illegible]
[illegible]
[illegible]
[illegible]

Modification Form for Permit BIO-UWO-0147

Permit Holder: Sung Kim

Approved Personnel
(Please stroke out any personnel to be removed)

Sarah Spanton

Andrew Makins

Soon Duck Ha

Additional Personnel
(Please list additional personnel here)

Anthony Bruni

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. *
Approved Microorganisms	P. aeuregenosa, S. typhimurium	
Approved Cells	Human (primary), rodent (primary), human (established), THP-1, rodent (established), RAW 2649	
Approved Use of Human Source Material	Blood (whole)	PARF - CFP mRFP - Rab7 mRFP - Rab5
Approved GMO		
Approved use of Animals	mice	

*PLEASE ATTACH A MATERIAL SAFETY DATA SHEET (MSDS) FOR ANY BIOHAZARDS.
PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT INCLUDES THE BIOHAZARDS THAT WOULD BE USED.

Date of last Biohazardous Agents Registry Form: Apr 28, 2008

Signature of Permit Holder:

Date: Apr 24, 08

Modification Form for Permit BIO-UWO-0147

Permit Holder: Sung Kim

Approved Toxin(s)		

Bio - UWO - 0147

THE UNIVERSITY OF WESTERN ONTARIO
BIOHAZARDOUS AGENTS REGISTRY FORM
Revised Biohazards Subcommittee: September, 2007

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario where the use of biohazardous infectious agents are described in the experimental work proposed. The form must also be completed if animal work is proposed involving the use of biohazardous agents or animal carrying zoonotic agents infectious to humans. Containment Levels will be required in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Health Canada (HC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Completed forms are to be returned to Occupational Health and Safety (Stevenson-Lawson Building, Room 60) for forward to the Biohazard Subcommittee. For questions regarding this form, please contact the Biosafety Coordinator at extension 84135. If there are changes to the information on this form (excluding grant title and funding agencies) modifications must be completed and sent to Occupational Health and Safety. See website: www.uwo.ca/humanresources

PRINCIPAL INVESTIGATOR Kim, Sung Ouk
SIGNATURE
DEPARTMENT Micro & Immuno
ADDRESS SDRI Rm 119, 1400 Western Rd.
PHONE NUMBER 84961
EMAIL sung.kim@schulich.uwo.ca

Location of experimental work to be carried out: Building(s) SDRI Room(s) 119

*For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to it being sent to Occupational Health and Safety (See Section 12.0, Approvals). For research being done at Lawson Health Research Institute, London Regional Cancer Centre, Child and Parent Research Institute or Robarts Research Institute, University Biosafety Committee members can also sign as the Safety Officer

GRANT TITLE(S):
~~- Dissecting signaling mechanisms of TLR- induced necrotic cell death~~
- Investigating the mechanisms of Nod-like receptor-induced cytokine release and cell death in macrophages

PLEASE ATTACH A BRIEF DESCRIPTION OF YOUR WORK, SUCH AS THE RESEARCH GRANT SUMMARY THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED. PROJECTS SUBMITTED WITHOUT A SUMMARY WILL NOT BE REVIEWED.

FUNDING AGENCY/AGENCIES CIHR

Names of all personnel working under Principal Investigator's supervision in this location:
Soon-Duck Ha
Andrew Martins
Antony Bruni
Sarah Spurstan

A DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED
Page 1 of 5

1.0 Microorganisms

1.1 Does your work involve the use of microorganisms or biological agents of plant or animal origin (including but not limited to viruses, prions, parasites, bacteria)? ☒ YES ○ NO
If no, please proceed to Section 2.0

1.2 Please complete the table below

Name of Biological agent(s)	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time?	Source/ Supplier	Health Canada or CFIA Containment Level
P. aeruginosa	☒ Yes ○ No	☒ Yes ○ No	○ Yes ○ No	2×10^9 cells		○ 1 ☒ 2 ○ 3
S. typhimurium	☒ Yes ○ No	☒ Yes ○ No	○ Yes ○ No	2×10^9 "		○ 1 ☒ 2 ○ 3
	○ Yes ○ No	○ Yes ○ No	○ Yes ○ No			○ 1 ○ 2 ○ 3
	○ Yes ○ No	○ Yes ○ No	○ Yes ○ No			○ 1 ○ 2 ○ 3

Please attach a Material Safety Data Sheet or equivalent from the supplier.

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ☒ YES ○ NO
If no, please proceed to Section 3.0

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture in the table below

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue
Human	☒ Yes ○ No	primary peripheral blood, ~~murine~~
Rodent	☒ Yes ○ No	peritoneal, bone-marrow derived macrophages
Non-human primate	○ Yes ○ No	
Other (specify)		

2.3 Please indicate the type of established cells that will be grown in culture in the table below.

Cell Type	Is this cell type used in your work?	Specific cell line(s)	Supplier / Source
Human	☒ Yes ○ No	THP-1	ATCC
Rodent	☒ Yes ○ No	RAW 264.7	ATCC
Non-human primate	○ Yes ○ No		
Other (specify)	○ Yes ○ No		

2.4 For above named cell type(s) indicate HC or CFIA containment level required ○ 1 ☒ 2 ○ 3

Please attach a Material Safety Data Sheet or equivalent from the supplier (for primary cells only)

DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? ✓ YES O NO
if no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	Source/Supplier /Company Name	Is Human Source Material Known to Be Infected With An Infectious Agent? YES/NO	Name of Infectious Agent (If applicable)	HC or CFIA Containment Level (select one)
Human Blood (whole) or other Body Fluid	Volunteers	✓ Yes O No		O 1 ✓ 2 O 3
Human Blood (fraction) or other Body Fluid		O Yes O No		O 1 O 2 O 3
Human Organs (unpreserved)		O Yes O No		O 1 O 2 O 3
Human Tissues (unpreserved)		O Yes O No		O 1 O 2 O 3
Human Organs (preserved)		O Yes O No		O 1 O 2 O 3

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents or cells described in Sections 1.0 and 2.0? O YES ✓ NO
if no, please proceed to Section 5.0

4.2 Will genetic sequences from the following be involved:

- HIV O YES O NO
if YES specify_____
- HTLV 1 or 2 or genes from any CDC class 1 pathogens O YES O NO
if YES specify_____
- Other human or animal pathogen and or their toxins O YES O NO
if YES specify_____

4.3 Will intact genetic sequences be used from

- SV 40 Large T antigen O YES O NO If YES specify_____
- Known oncogenes O YES O NO If YES specify_____

4.4 Will a live viral vector(s) or bacterial plasmid be used for gene transduction O YES O NO
If YES name _____
Please attach a Material Safety Data Sheet or equivalent

4.5 List specific vector(s) to be used _____

4.6 Will vectors be replication defective O YES O NO

4.7 Will vectors be infectious to humans or animals O YES O NO

4.8 Will cells be propagated to increase the Containment Level required O YES O NO

DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials using the viral vector in 4.0 be conducted? O YES O NO
If no, please proceed to Section 6.0
If YES attach a full description of the make-up of the virus.

5.2 Will virus be able to replicate in the host? O YES O NO

5.3 How will the virus be administered? _____

5.4 Please give the Health Care Facility where the clinical trial will be conducted: _____

5.5 Has human ethics approval been obtained? O YES O NO O PENDING

6.0 Animal Experiments

6.1 Will any of the agents listed be used in live animals? ✓ YES O NO
If no, please proceed to section 7.0

6.2 Name of animal species to be used mouse, (57) BL/6

6.3 AUS protocol # pending

6.4 If using murine cell lines, have they been tested for murine pathogens? ✓ YES O NO

7.0 Use of Animal species with Zoonotic Hazards

7.1 Will any of the following animals or their organs, tissues, lavages or other bodily fluids including blood be used.

- Pound source dogs O YES ✓ NO
- Pound source cats O YES ✓ NO
- Cattle, sheep or goats O YES ✓ NO
- Non- Human Primates O YES ✓ NO If YES specify species _____
- Wild caught animals O YES ✓ NO If YES specify species _____ colony # _____
- Birds O YES ✓ NO
- Others (wild or domestic) O YES O NO

8.0 Biological Toxins

8.1 Will toxins of biological origin be used? O YES ✓ NO
If no, please proceed to Section 9.0

8.2 If YES please name the toxin _____

8.3 What is the LD_{50} (specify species) of the toxin _____

8.4 Please attach information such as a Material Safety Data Sheet, for the toxin(s) used

DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED

9.0 Import Requirements

9.1 Will the agent be imported? ✓YES O NO
If no, please proceed to Section 10.0
If yes, country of origin USA

9.2 Has an Import Permit been obtained from HC for human pathogens? ✓YES O NO

9.3 Has an import permit been obtained from CFIA for animal pathogens? ✓YES O NO

9.4 Has the import permit been sent to ORS? O YES ✓NO
If yes, Permit # .....

10.0 Training Requirements for Personnel named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by ORS

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS
- Employee Health and Safety Orientation

As the Principal Investigator I have ensured that all of the personnel named on the form who will be using any of the biohazardous agents in Sections 1.0 to 9.0 have been trained.

SIGNATURE .....

11.0 Containment Levels

11.1 For the work described in sections 1.0 to 9.0 please indicate the highest HC or CFIA Containment Level required O 1 ✓2 O 3

11.2 Has the facility been certified by ORS for this level of containment? ✓YES O NO

11.3 If yes, please give the date and permit number Bio-uwo-0142

12.0 Approvals

UWO Biohazard Subcommittee

Signature ..... Date 28 Apr 08

Signature of Supervisor (where Principal Investigator is a student) .....

Signature ..... Date 25/04/08

Signature of Chair, Senate Committee on Animal Use .....

Apr 23, 2008