Gene tic Screens

1. interesting phenotypes
2. Forward
3. Reverse
MATING TYPE IN S. Cerevisiae

1. Each produce α-factor (produce α-factor)
2. Each mating-type has a receptor for the peptide

MATα × MATα

MATa MATa

MATa

Diploid

1. cannot mate
2. enter meiosis → sporeulate.

A. Mating is stable in lab strains (ho-)
B. Wild-type strains can switch mating-types (H0)

Four meiotic

○ ○ can mate with sibs

B. Ask about the pattern
test with α-factor as it will cause MATα to arrest in G1 but not MATα

1. Mothers switch
   2. Pairs of cell switch — act between G1 + S
   3. Most but not all mother switch

c. Is there exchange of information?

   use mata− mutant
   mata− mutant

   "Mutants are healed by switching"

D. Cloning the genes

1) complementation

HML [ ] MAT [ ] HMR [ ]

HMR, HML are "silent" copies of mating-type information
MATα ≠ MATα they encode different proteins

MATα encodes Matα1
MATα encodes Matα2
MATα encodes Matα1

Three sets of genes are regulated

α-specific asg
α-specific xsg
haploid-specific hsq

HML
α

MAT

HMR
α

a = △

α₁, α₂ → WHA1

α₁, α₂

α-specific
α-facor
α-specific

α₂ → aspecific

a/x

α₁ + α₁ → MATα α/x specific
How are HMRa and HMLa kept silent?

Rine + Herskowitz

cis-acting sites not present at MAT
E = essential I = important

1. Turn on HMR, HML → sterile
   α-factor resistant
Screen

\[ \overset{\text{\(m\alpha r\)}}{\alpha} \rightarrow \overset{\text{\(m\alpha r\)}}{\alpha} \]

\[ \overset{\text{\(m\alpha r\)}}{\text{\(m\alpha r\)}} \text{ mate as } \alpha \text{ cells as diploids} \]

\[ \underset{\text{\(M\alpha\)}}{\text{\(M\alpha\)}} \]

\[ \overset{\text{\(m\alpha r\)}}{\text{\(m\alpha r\)}} \text{ mate as } a \text{ cells} \]

\[ \overset{\text{\(H\alpha \text{r} \ m\alpha \text{r}\)}}{\text{\(H\alpha \text{r} \ m\alpha \text{r}\)}} \text{ \(h\alpha \text{r} \text{r} \ m\alpha \text{r}\)} \text{ mate as } \alpha \text{ cells} \]

screened 675,000 colonies for mating as \(\alpha\) cell \(\rightarrow\) 296

\[ \text{SIR1} \quad 73 \]

\[ \text{SIR2} \quad 13 \quad \text{deacetylase} \]

\[ \text{SIR3} \quad 31 \quad \text{interacts w/ sir2, sir4, histone tails} \]

\[ \text{SIR4} \quad 24 \quad \text{recruited by Rap1} \]

\[ \text{Unknown} \quad 17 \]
sir2, sir3, sir4  →  non-mating  
derepression of HMR, HML

sir1  →  "leaky" still able mate  
null alleles are "leaky"

generically equivalent cells have different phenotypes.

look at phenotype of single cells

α-factor medium

a  no division - sensitive  ◇ shmoo
a  division  - resistant  0.88
a/d  resistant
a  sir2  resistant
a  sir3  resistant
a  sir4  resistant
a sir1-1  20 S : 80 R
a  sir1Δ  21 S : 79 R
a  sir1Δ + SIR1  resistant

Questions
1) threshold response
2) ask if there only two types of cells

◇ return to medium  ◇
   ↓ without α-factor

◇ return to α-factor
4 x 10^{-3} \text{ change in sterile / cell division)}

MOTHERS SWITCH AND DAUGHTERS DO NOT

1. Mothers transcribe HO gene
   - HO endonuclease that cuts
   - double stranded break
   - gene conversion using HMR, HML as templates

2. What allows mothers (or prevents daughters) to transcribe HO?
   a. regulator of HO transcription
      - SWI4- SWI6 complex
      - SWI5
   b. SWI5 is transcribed only in S, G2, M and constitutive expression allows daughters

5 genes required for HO expression → SNF
c. SWI5 is found in both M and D's

d. what activates SWI5 in mothers?

**Genetic Screen for daughters that switch**

1. HO+ ste3Δ (cortex gene) for α-factor

   mutagenize

   α-factor agar

   wild-type mut

   \[ \alpha \]

   \[ \alpha \]

   \[ \alpha \]

   \[ \alpha \]

   arrest with 4 shmoos

   arrest

   shmoos + budding cells

   \[ \text{ash1} \]

1. show ash1 is recessive
2. clone by complementation - GATA transcription factor
3. daughters transcribe HO
4. Ash1 protein is only in daughters nucleus
5. O/E \( \rightarrow \) inhibit switching in mothers
6. Ash1 mRNA is localized to daughters
Localization of Ash1 mRNA

a. looked for defective URS1 function

\[
\begin{align*}
\text{Ho} & \quad \text{Ade2} & \quad \text{Ho active} \\
\text{Ho} & \quad \text{Can1} & \quad \text{Canavamine S}
\end{align*}
\]

↓

look for inactive Ho promoter

\[
\begin{align*}
\text{urs1 urs2} & \quad \text{red can}^R \\
\text{urs1 urs2} & \quad \text{can}^R
\end{align*}
\]

\[
\begin{align*}
\text{Swi-Snf complex} & \quad \text{Chromatin remodeling complex.}
\end{align*}
\]

add 3'rd gene

\[
\begin{align*}
\text{urs2 GAL-HO} & \quad \text{Loc2 gal1}
\end{align*}
\]

direct mother specific Ho

1) dependant all SWI/SNF genes except SWI5

\[
\begin{align*}
\text{3000 can}^R \text{ red colonies}
\end{align*}
\]

\[
\begin{align*}
\text{315} & \quad \text{gal}^1
\end{align*}
\]

222 swi5 mutants characterized 46 \rightarrow 5 comp. groups
<table>
<thead>
<tr>
<th>SHE1</th>
<th>Myosin 4 (class V myosin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHE5</td>
<td>BNII (formin)</td>
</tr>
<tr>
<td>SHE2</td>
<td>RNA binding protein, interacts with SHE3</td>
</tr>
<tr>
<td>SHE3</td>
<td>Adapter between SHE2 and MYO4</td>
</tr>
<tr>
<td>SHE4</td>
<td>Myosin binding domain</td>
</tr>
</tbody>
</table>

mRNAs move along actin cables to the daughter bud.
Mother and Daughter Are Doing Fine: Asymmetric Cell Division in Yeast

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The underlying principle of every developmental program is that single cells divide to give rise to daughter cells with different developmental fates. This is true not only for the fertilized egg that develops into an adult, but also for the yeast cell that buds. In general, two mechanisms can account for this phenomenon. Differences between daughter cells may originate from differences in their environment. For example, vulva formation in Caenorhabditis elegans requires cell–cell interaction and signaling between equipotent cells (reviewed by Kenyon, 1995). Alternatively, differences between daughter cells can be generated by unequal segregation of cell fate determinants during cell division. Examples of this process include cell division in the bacterium Caulobacter crescentus, whereby a nonmotile stalked cell divides into a motile swarmer cell and another stalked cell, spore formation in Bacillus subtilis (reviewed by Shapiro, 1993), and sensory organ formation in Drosophila melanogaster (reviewed by Ryhu and Knoblich, 1995).

One of the best studied examples of asymmetric segregation of cell fate is mating-type switching in the budding yeast S. cerevisiae. Haploid yeast cells exist in either of two mating types, a or α. Mother cells, defined as cells which have budded in the previous cell cycle and have thus given birth to a daughter cell, typically switch their mating type (Figure 1A). Daughter cells, on the other hand, do not switch mating type. This results in a close apposition of cells of opposite mating type and thus promotes mating and the formation of a/a diploid cells (Figure 1B; reviewed by Herskowitz, 1988; Nasmyth, 1993). Mating-type switching is ultimately accomplished by a gene conversion event in which a- or α-specific sequences at the mating-type locus (MAT) are replaced by information specifying the opposite mating type, which resides in a transcriptionally silent region elsewhere in the genome (reviewed by Nasmyth, 1993; Herskowitz et al., 1992). The Ho endonuclease initiates this mating-type interconversion and, consistent with this role, is expressed only in mother cells (Nasmyth, 1983). Restriction of Ho activity to mother cells is largely if not solely determined by the pattern of HO transcription. The gene is transiently transcribed only in mother cells and only late in the G1 phase of the cell cycle. Thus, determining how HO gene expression is confined to mother cells seems to be the key to understanding why only mother cells switch mating type.

Two regions in the HO promoter are responsible for restricting HO expression to the late G1 phase of the cell cycle and to mother cells. URS2 (upstream regulatory sequence 2) confines HO expression to late G1, whereas URS1 is necessary for mother cell specificity. At least ten regulatory factors, designated Swi1p–Swi10p, have been identified that are required for correct expression of the HO gene. Among these, Swi4p and Swi6p confer cell cycle regulation through URS2 (reviewed by Koch and Nasmyth, 1994) and the zinc finger transcription factor Swi5p activates HO transcription in mother cells via the URS1 element (Tebb et al., 1993, and references therein). None of these regulatory factors, however, seems to be the primary or sole determinant for the asymmetric expression of the HO gene. At first, Swi5p had appeared to be a promising candidate because Swi5 is required for HO expression in mother cells and because either constitutive expression of Swi5 (Lydall et al., 1991) or the stabilization of Swi5p by mutation (Tebb et al., 1993) causes a certain fraction of daughter cells to switch mating type. However, Swi5p is found in both mother and daughter cells (Figure 2A) and activates transcription of genes other than HO equally well in both mother and daughter cells (Koch and Nasmyth, 1994).

Thus, some other factor, either a mother cell–specific activator or a daughter cell–specific repressor, must exist to generate the asymmetric expression of HO.

In this issue of Cell, the Herskowitz (Sil and Herskowitz, 1996) and Nasmyth (Bobola et al., 1996) groups report an important advance in understanding how HO asymmetry is generated: they have identified a negative regulator of HO expression that is daughter cell specific. This factor, termed Ash1p for asymmetric synthesis of HO, preferentially accumulates in the daughter cell nucleus. Findings by Jansen et al. (1996) (this issue of Cell) may provide a clue as to how Ash1p becomes localized in this way: an unconventional myosin is required for the preferential accumulation of Ash1p in daughter cells.

Figure 1. The Pattern of Mating-Type Switching in Budding Yeast
(A) Pattern of mating-type switching. Mother (M) but not daughter (D) cells generate two descendant cells with switched mating types.
(B) Mating-type switching facilitates zygote formation.
Figure 2. Temporal and Spatial Localization of Swi5p, Ash1p, She1p/Myo4p, and She3p
(A) During G2, SWI5 RNA synthesis commences and the protein accumulates in the cytoplasm. Shortly after completion of anaphase, the protein enters the nucleus, where it is unstable. By late G1, most but not all Swi5p has been degraded (indicated by light red; Tebb et al., 1993).
(B) Ash1p is absent during early stages of the cell cycle, but shortly after completion of anaphase it preferentially accumulates in the nucleus located in the bud. The protein remains present in G1 daughter (D) cells, but rapidly disappears as daughter cells enter the cell cycle.
(C) She1p/Myo4p and She3p are evenly distributed in early G1, but shortly before entry into the cell cycle they accumulate in a patch, perhaps marking the presumptive bud site. Until late anaphase, the proteins are predominantly localized to the growing bud. Shortly prior to completion of anaphase, the protein becomes evenly distributed between mother (M) and daughter (D) cell.

suggesting that actin-based transport may play a role in generation of HO asymmetry.

Three Mutant Hunts Converge on ASH1
Three mutant hunts led to the identification of ASH1. Although conceptually different, all three were based on the same hypothesis: a daughter cell–specific repressor might be responsible for asymmetric HO expression, so that inactivation of such a repressor would lead to expression of HO in daughter cells, allowing them to switch mating type.

The screen employed by Sil and Herskowitz (1996) took advantage of the fact that cells exhibit a visible morphological change when exposed to mating pheromone secreted by cells of opposite mating type. In response to a factor pheromone, a cells stop dividing and differentiate into gametocytes that form a mating projection (known as a shmoos). Cells respond to a factor pheromone in the same way. Sil and Herskowitz (1996) carried out a direct screen for daughter cells that can switch mating type. Under conditions in which wild-type cells form microcolonies containing both shmoos and budded cells, these mutants were identified because they form distinctive microcolonies containing four shmoos.

Bobola and coworkers (1996) performed two separate genetic selections to identify genes involved in establishing HO asymmetry. In the first selection, they generated a fusion between the HO promoter and a gene essential for cell division, the CDC6 gene. The HO promoter restricts expression of CDC6 to mother cells. Thus, cells whose endogenous CDC6 gene has been replaced by an HO–CDC6 fusion cannot form colonies because daughter cells lack this essential gene product. However, mutant cells that express HO inappropriately in daughter cells should divide and form a colony.

The rationale for their second mutant hunt was based on findings by Jansen et al. (1996), who identified five new genes, SHE1–SHE5 (for Swi5p–dependent HO expression), essential for HO expression in mother cells. The expectation was that the She proteins might localize to the mother cell. However, SHE1 encodes an unconventional myosin, which surprisingly preferentially accumulates in the growing bud—the future daughter cell—and not in the mother cell. To explain this paradoxical finding, Bobola et al. (1996) postulated that she1 mutants are defective in restricting a negative regulator of HO expression to daughter cells. According to this model, inactivation of such a negative regulator should allow she1 mutants to express HO. Bobola et al. (1996) therefore looked for mutations that restore HO expression in a she1 mutant background.

ASH1 Is Required for Restricting HO Expression to Mother Cells, and Ash1p Preferentially Accumulates in Daughter Cells
The three screens described above identified alleles of a single gene, ASH1. Excitingly, ASH1 fulfills all of the criteria for being the key determinant of asymmetric HO expression. First, ASH1 is necessary for preventing daughter cell switching. Cells deleted for ASH1 express HO in daughter cells, which leads to an increase in the frequency of mating-type switching from less than 1% to approximately 90% (Bobola et al., 1996; Sil and Herskowitz, 1996). Furthermore, overexpression of ASH1 is sufficient to reduce mating-type switching in mother cells (Sil and Herskowitz, 1996). Most importantly, Ash1p activity is restricted to daughter cells. Bobola et al. (1996) and Sil and Herskowitz (1996) show that the protein preferentially accumulates in daughter cells (Figure 2B). After completion of anaphase, Ash1p accumulates in the nucleus located in the bud—the future daughter cell—where it remains present during G1. The protein rapidly disappears as daughter cells enter the cell cycle and appears to be absent during S phase, G2, and mitosis. This asymmetric accumulation of Ash1p is exhibited by 75% of cells (Sil and Herskowitz, 1996) or more (Bobola et al., 1996). Interestingly, low levels of Ash1p are also observed in 25% of mother cells. This observation might explain why in wild-type cells only 70% of mother cells switch mating type, whereas in ash1 mutants mother cells the frequency of switching is increased to 100%. Residual Ash1p in some wild-type mother cells might prevent HO expression.

Mechanisms of HO Repression
Ash1p contains a domain with homology to the zinc finger domain of the GATA-like transcription factor family. It is thus conceivable that Ash1p binds to sequences within the HO promoter, thereby inhibiting transcriptional activation. Genetic data support the view that Ash1p antagonizes Swi5p first, when Swi5 is deleted in ash1 mutants, daughter cell switching drops to minimal levels, showing that this switching is dependent on SWI5. Second, the phenotypes associated with modulating the levels of SWI5 are exactly opposite those of ASH1. Deletion of SWI5 causes loss of HO expression.
in mother cells, whereas loss of ASH1 function causes both mothers and daughters to express HO. Moreover, constitutive expression of SWI5 allows daughter cells to switch (Lydall et al., 1991), whereas overexpression of ASH1 blocks HO expression (Bobola et al., 1996) and mating-type switching in mother cells (Sil and Herskowitz, 1996). Finally, the accumulation of Ash1p in daughter cell nuclei transiently coincides with that of Swi6p (compare Figures 2A and 2B). Interestingly, deletion of a particular domain of Swi6p allows daughter cells to switch mating type (Tebb et al., 1993). Ash1p may interact with this region of Swi6p and thereby inhibit Swi6p-mediated activation of the HO promoter.

**Cell Polarity May Translate into Developmental Asymmetry**

Confining Ash1p activity to daughter cells is clearly important for restricting HO expression to mother cells. But how is Ash1p distribution regulated? The SHE genes identified by Jansen et al. (1996) in a selection for potential mother-specific activators of HO expression provide an important clue: SHE1, SHE2, SHE3, and SHE5 (SHE4 has not been tested) are required for the preferential accumulation of Ash1p in daughter cell nuclei at the end of anaphase. Deletion of any of these genes results in a nearly symmetric distribution of Ash1p in mother and daughter cells (Bobola et al., 1996) as well as the failure of mother cells to switch mating type (Jansen et al., 1996). SHE genes are therefore essential for preventing accumulation of Ash1p in mother cells, which is crucial for HO expression. SHE2, SHE3, and SHE4 encode novel proteins, and SHE3 is allelic to BN1, a gene thought to be involved in cytokinesis. SHE1 is allelic to MYO4, a member of the class V unconventional myosin family (reviewed by Moosiker and Cheney, 1995).

Owing to its mode of cell division (budding), the S. cerevisiae cell is structurally asymmetric. The actin cytoskeleton has been implicated in generation of this inherent asymmetry (reviewed by Lew and Reed, 1995). The localization pattern of She1p/Myo4p and She3p is remarkably similar to that of actin and proteins involved in budding and cell polarization: both proteins are cytoplasmic and preferentially accumulate in the bud, the future daughter cell (Figure 2C). The actin-based motor Myo2p, a close relative of She1p/Myo4p, transports vesicles on asymmetrical actin filaments into the growing bud (Moosiker and Cheney, 1995). She1p/Myo4p, and perhaps other She proteins, could facilitate accumulation of Ash1p in daughter cells in a similar way. Thus, they might contribute the structural asymmetry of the actin cytoskeleton to a developmental asymmetry, the asymmetric accumulation of the mating-type switching determinant Ash1p.

**Possible Mechanisms for the Generation of Ash1p Asymmetry**

Restriction of HO expression to mother cells depends largely, if not solely, on asymmetric accumulation of Ash1p in the daughter cell nucleus. Thus, the key to understanding HO asymmetry lies in understanding how Ash1p asymmetry is generated. Although we do not know the answer, several mechanisms can be envisioned that bring about this asymmetry. In a process similar to that used for asymmetric localization of morphogens in the Drosophila embryo (St. Johnston, 1995), ASH1 RNA or protein might be localized to the daughter cell nucleus. In this model, the potential actin-based motor She1p/Myo4p would facilitate transport of ASH1 RNA or protein to the future daughter cell via polar actin filaments.

Alternatively, as yet unidentified factors promoting accumulation of Ash1p in daughter cells might be transported into the bud by She1p/Myo4p and perhaps other She proteins. Such factors might be required to stabilize Ash1p in daughter cells or to promote ASH1 RNA translation. Indeed, translational control is an important mechanism for spatial restriction of cell fate determinants in many organisms (reviewed by Curtis et al., 1995). Probably the best example of such a mechanism is the regulation of the posterior cell fate determinants nanos and oskar in D. melanogaster. Both RNAs are translated only when localized to the posterior pole of the oocyte (Curtis et al., 1995). Analogously, ASH1 RNA could be translated only in daughter cells because factors required for its translation are localized specifically to daughter cells by She proteins.

Another possibility is that ASH1 is transcribed only in daughter cells and that She proteins are required to restrict transcription of ASH1 to daughter cells. Interestingly, ASH1 transcription requires SWI5 (Bobola et al., 1996), which is also required for mother cell-specific expression of HO. This finding explains why Ash1p accumulates only after completion of anaphase, at the time when Swi6p enters the nucleus (Figure 2A). Swi6p activity, however, is not asymmetric. Thus, if ASH1 transcription is daughter cell-specific, other factors must exist that confer asymmetric expression of ASH1. She proteins could facilitate transport of such factors into daughter cells.

**Coupling Temporal and Spatial Control at the HO Promoter**

Unlike other SWI5-dependent transcripts, which are transcribed as cells exit mitosis, activation of HO expression is delayed until the late G1 phase of the next cell cycle. Thus, temporal as well as spatial regulation might be important for restricting HO expression to mother cells. Sil and Herskowitz (1996) and Bobola et al. (1996) propose a model that incorporates both notions: as cells exit mitosis, Swi6p enters the nucleus of both mother and daughter cells, where it activates transcription of ASH1. Asymmetric accumulation of Ash1p in the daughter cell nucleus brought about by She proteins therefore coincides with entry of Swi6p into the nucleus (compare Figures 2A and 2B). In daughter cells, Ash1p inhibits Swi6p function or interferes with activation of HO transcription (both possibilities are indicated by a question mark in Figure 3). Although Ash1p is absent in mother cells, Swi6p cannot activate HO transcription because it must await Swi4p and Swi6p function, which confines HO transcription to the late G1 phase of the cell cycle. This temporal delay in activation of HO by Swi4p and Swi6p might provide a time window in which Ash1p can accumulate in the daughter cell nucleus to levels sufficient to inhibit all Swi6p function or to prevent binding of transcriptional activators to the HO promoter (Sil and Herskowitz, 1996). This could lead to repression of HO expression in daughter cells (Figure 3).
In 1979, Strathern and Herskowitz showed that mother and daughter cells differ in their competence to switch mating type. The first breakthrough in understanding mother-daughter specificity of mating-type switching was the finding that the HO gene is expressed only in mother cells (Nasmyth, 1983). Now, the identification of a daughter cell-specific repressor of HO expression might well have solved the puzzle of how HO expression, and thus mating-type switching, is confined to mother cells. It seems, however, that ASH1 is not all that different from HO: it too accumulates preferentially in one of the two descendant cells—this time in the daughter cells. Although the origin of this asymmetry is not understood in detail, actin-dependent transport may be responsible. In the Drosophila oocyte, polarized microtubules and the actin cytoskeleton are thought to provide a structural basis for localizing cell fate determinants (reviewed by Lehmann, 1995; Erdélyi et al., 1995). Perhaps the polarized actin cytoskeleton plays a similar role in facilitating localization of Ash1p. Utilizing cytoskeletal asymmetry to generate developmental asymmetry may prove to be a common mechanism by which a cell generates two daughter cells of different fates.

Selected Reading


UPR — unfolded protein response

1. adapt to ER stress via a transduction pathway to match the "folding capacity" of the ER to its need

2. UPR \rightarrow \text{increase ER membranes}
   \text{increase chaperones}
   \text{increase protein modification enzyme}
   \text{decrease translation}
   \text{decrease entry of proteins into ER}
   \text{increase targeting of unfolded protein for degradation (ERAD)}

I. Three important components
A.
   a. \text{IRE1} \quad \text{transmembrane kinase}
   b. \text{HAC1} \quad \text{bZIP TF}
   c. \text{RL61} \quad \text{tRNA ligase}

1. This pathway is activated in
   a. multiple cancers
   b. CFTR
   c. Alzheimer's
   d. Viral infections
   e. Sleep deprivation
B. Genes controlled

UPR controls transcription of a set of genes (chaperones and phospholipid biosyn).

1) BiP (GRP78) KAR2
PDI

GRP 170
GRP 94
ERP 72
GRP 58

induced by glucose starvation
impair glycosylation and cause protein misfolding
Mammals

FKBP2
LHS1
EUG1 (Pch1-like)
ERO1
Yeast

*KAR2 is induced when unfolded protein accumulate in ER

2) Genes in yeast share a 22bp motif (UPRE)
Genes in mammals share a 28bp motif

\[ \text{UPRE} \rightarrow \text{repressor} (\beta\text{gal}) \rightarrow \text{by ER stress} \]

Isolation of mutants

wild-type cells \(\rightarrow\) replica plated \(\rightarrow\) screen for white colonies.

\(+\) x-gal + tunicamycin (fail to turn on \(\beta\text{-gal}\))
Secondary screen
- discard tunicamycin resistant mutants
- sensitive to UPR by 2-deoxyglucose
- inability to induce a secondary promoter
- failure to make KAR2 mRNA

2/45,000 colonies pass all tests

Genetic tests

a) recessive
b) 2:2 segregation
c) complementation by genomic library of high copy number plasmid
d) tested inserted for single copy rescure.
e) insert was 9kb
f) make null allele

g) Gene identified was already called
   \[ \overline{IRE1} \rightarrow \text{inositol auxotroph} \]

![Diagram of signal transduction pathway]

ERlumin

Cytoplasm

Other alleles show intra-allelic complementation
K702R / \[ AC\text{tail (133aa)} \rightarrow \text{WT (kinase domain)} \]
Genetic screen for more UPR pathway components

UPRE - UPRE - UPRE - UPRE — [β gal]

(4 copies) 43x as sensitive as 1 copy

ΔIRE1 4xUPRE - HIS3

Screen multicopy library for plasmids that increase HIS3 expression

20 plasmids
IRE1
SWI4 > TF
HAC1 βZIP

Genetic tests
1. Do deletions of SWI4 or HAC1 change KAR2 expression / UPR

ΔSWI4 no effect on UPR = WT
ΔHAC1 no UPR = ΔIRE1

2. Gel shift shows Hac1p binding to UPRE

3. Hac1p is only present when prefins (UPR) accumulate in ER
5) Regulation of Hac1 expression

a) no Hac1 protein is detected in yeast in the absence of UPR.

b) transcription of HAC1 independent of UPR

* c) Post-transcriptional regulation

6) HAC1 mRNA

```
5' UTR exon || exon UTR AAA
```

a) mRNA in uninduced cells

```
UTR exon *** exon UTR AAA
```

b) mRNA in induced cells: SPliced

c) this form is translated and activates UPR

d) this form has very short half-life
4. HAC1 mRNA is spliced in response to UPR

- Naa
- unspliced
- 180aa
- spliced

- make Ab to spliced C terminus

- spliced w/o mRNA
- constitutive Hac1p production
- that IRE1-independent

5. Splicing
   a. splice junctions are not conserved
   b. ts splicing mutant do not affect HAC1 splicing

Summarize:

UPR (+Tunicamycin) → Irel actuation

↑ ER → HAC1 binding ← HAC1 splicing
to UPRE DNA
**Synthetic lethal screen**

- **3p2. (KAR2 in yeast)** gene used for SL

  a) HDEL motif needed to retain proteins in ER
  
  \[
  \text{Kai2-HDEL} \rightarrow \text{induce UPR since Kai2 is secreted from ER}
  \]

  b) previous lethad analysis showed \( \text{ile1} \, \Delta \, \text{Kai2} \, \Delta \text{HDEL} \)
  is lethal

  c) starting strain \( \text{Kai2} \, \Delta \text{HDEL} \, \Delta \text{ile} \)
  ade3
  
  plasmid with \( \text{ADE3} \, \text{KAR2} \)

  d) isolated \( 17 \div 20,000 \) non-sectoring colonies

  e) 2\(^{nd}\) assay
  1. induction of \( \text{UPRE} \, - \beta \text{gal reporter} \)
  2. \( 3/17 \)

  f) complementation assay with \( \text{ile1} \)
  
  \( 2/3 \) fail to complement \( \text{ile1} \)
  
  \( 1/3 \) \( \text{rg} \, 1-100 \)

  g) identify gene by complementation of mutant phenotype with low copy number genomic library.

  complementation of which phenotype?
  
  \( \text{inositol} \)

  h) \( \text{RLG1} \) \( \rightarrow \) tRNA ligase
SIDE NOTE:
1. A flgi - lethal
2. Three activities
   a. polynucleotide kinase, cyclic phosphodiesterase, a denylate synthetase, RNA ligase
   \[ \text{tRNA} + \text{INS} \xrightarrow{\text{endonuclease}} \text{ligase} \]
   \[ \text{ligase} \]

i. sequenced mutant allele -> his -> tyr change

4) Does tRNA ligase play a role in HAC1 splicing?

- Northern blots of HAC1
  - ire1
  - rgl1-100
  - ire1 rgl1-100
  - cen
  - ire1
  - rgl1-100

*transcribed
under no UPR

- very low

a) control is ACTIN1, a gene that is spliced by the spliceosome

b) test if mutantless HAC1 is stable.

c) epistasis test

\[ \text{IRE1} \xrightarrow{} \text{HAC1} \]
\[ \text{HAC1} \xrightarrow{} \text{IRE1} \]
\[ \text{IRE1, HAC1} \xrightarrow{} \text{IRE1, HAC1} \]

\[ ? \]
5) what kind of allele is reg1-100?

   a) partial loss of tRNA splicing

   OR

   b) pathway specific (separation of function allele)

   examine by two-dimensional gels

   [Diagram showing two-dimensional gel analysis]

   10% polyacrylamide + 4M urea
   20% polyacrylamide
   TM Urea

   reg1Δ + pGAL-RLG1 (+ galactose)  Δreg1 + pGAL-RLG1
   (+ dextrose)

   no tRNA haloes

   reg1-100 does not block tRNA splicing

   reg1-100 "separation of function allele"

6) do spliceosome mutations affect HAC1 splicing?

   prp2ts and prp8ts → HAC1 splicing occurs
   at restrictive temperature

   - tRNA ligase
   - not normal splicing
Figure 2
Mechanism of Ire1-mediated mRNA splicing in yeast. Unfolded proteins are recognized by the ER-luminal domain of Ire1, leading to clustering of this stress sensor in the ER membranes. The Ire1 cytosolic domains become juxtaposed, in turn promoting transautophosphorylation by the kinase domain (K) and concomitant activation of the cadoribonuclease domain (R). Base-pairing between the 5' UTR and the intron of HAC1 mRNA inhibits its translation; ribosomes are already loaded on the translationally inhibited mRNA. Ire1 excises the HAC1 mRNA intron, and the resulting exons are ligated by tRNA ligase. Spliced HAC1 mRNA is efficiently translated, producing the transcription factor Hac1, which travels to the nucleus and activates its target genes.
Fig 1. Model of the budding yeast kinetochore. Six subcomplexes (present in varying copy numbers), the Mif2/CENP-C protein and the Cse4/CENP-A centromeric histone variant assemble into a proposed kinetochore structure that links MTs to chromosomes.
Adapted with permission from Joglekar, A.P., K. Bloom, and E.D. Salmon

Fig 2. Kinetochore particles can be isolated by affinity purification of Dsn1-FLAG. Core kinetochore proteins co-purify with Dsn1-FLAG as visualized by silver-stained SDS-PAGE. Asterisk indicates non-specific copurifying proteins.
Daml complex

Duo1 - over-expression screen

Daml1 - synthetic lethal with mps1-1

ASK1 purified
DAD1 Two hybrid with Duo1
DAD2 purified
DAD3 purified
DAD4 purified
HSK3 multicopy suppressor of ask1
SPC19 src/purified
SPC34 src/purified