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FOREWORD

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*Helen Davis Keller*

PI - Signature

Date

**Annual Report for Grant Number DAMD17-94-J-4293, October 1997-September 1998.**

**Tumor Suppressor Genes in Early Breast Cancer and its Progression**

**Helen Donis-Keller, Ph.D.**

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## Introduction

### A. Response to Reviewer of 1997 Progress Report

We very much appreciate the careful consideration of our report by the reviewer. The reviewer for the 1997 report did not have any specific criticisms or technical issues which would be addressed in this section of the 1998 progress report.

### B. Nature of the problem

An increasing percentage of breast cancer is being detected at a pre-invasive stage: ductal carcinoma *in situ* (DCIS). DCIS is a form of breast cancer in which malignant cells have not penetrated the basement membrane (1). The histopathological diagnosis encompasses a heterogeneous group of subtypes, including comedo, cribriform, solid, papillary and micropapillary, some of which may differ in biological behavior. The potential for associated micro invasion and likelihood of recurrence after breast conservation therapy are higher with the comedo subtype (2, 3). As with invasive breast cancer, DCIS can also be stratified by nuclear grade: high, intermediate, and low. Circumstantial evidence that DCIS is a precursor lesion to invasive ductal carcinoma is based on three observations: the frequent co-existence of DCIS and invasive cancer in the same breast (4); the greatly increased risk of subsequent invasive breast cancer in women with biopsy-proven DCIS (5); and the finding that when a local recurrence is seen after breast-conserving treatment of DCIS there is a 50% chance that the recurrence will be of the invasive variety (6). DCIS is not an obligate precursor however, and other possible pathways to invasion may exist such as the *de novo* transition to malignancy of normal epithelium without an intervening non-invasive stage. For many years the standard treatment for DCIS has been total mastectomy, though lumpectomy with adjuvant radiation is being utilized currently for small, well localized areas of DCIS.

Lobular carcinoma *in situ* (LCIS), on the other hand, is not thought to be a pre-invasive cancer but rather an indicator of increased risk of breast cancer. Interestingly, the risk is the same in both breasts regardless of the side in which the LCIS was detected. That the LCIS cells do not inevitably progress to invasive breast cancer is evidenced by the fact that, of those cancers which do develop, half are of the invasive ductal variety (7).

Atypical lobular hyperplasia (ALH) and atypical ductal hyperplasia (ADH) are considered to be high-risk lesions both associated with an increase of 4-5 fold compared to the general female population. If a strong family history of breast cancer exists, the risk is doubled to 8 to 9 fold (5).

Our studies have concentrated on the genetic changes which occur in DCIS and the transition from DCIS to invasive breast cancer. A better understanding of the oncogenesis of breast cancer at the molecular level, and the correlation of this information with clinical data, may aid in treatment choices.

### C. Background of Previous Work

Most solid tumors arise due to the inactivation of tumor suppressor genes and activation of oncogenes. The accumulation of genetic changes is believed to result in the invasive followed by the metastatic phenotypes. Loss of heterozygosity (LOH) of one of a pair of alleles in tumor tissue compared to matched normal control can reveal areas of chromosome deletion which are likely to contain putative tumor suppressor genes. A large number of articles have been published on LOH in invasive breast cancer, and virtually every human chromosome has been shown to exhibit allelic loss (8,9). The most frequent losses in invasive breast cancer are seen on chromosome 7q (0-83%) (8,10,11), 16q (32-63%) (8,9,12-14), 17p (31-75%) (8,9,15-18), 17q (24-79%) (8,9,19-25), and 18q (24-69%)

(8,22,26,27). Less frequent losses are found on 1p (3-47%) (8,9,28), 1q (16-32%) (8,9,29,30), 3p (11-47%) (8,9,17), 6q (9-48%) (8,9), 8p (27-33%) (8,16) 11p (10-41%) (8,31) and 13q (16-40%) (9,17).

Several investigators have reported two distinct regions of loss on 8p in breast cancer, located at 8p21 and 8p22. Yaremko et. al. studied 20 examples of invasive ductal cancer and found the overall rate of LOH on 8p to be 55% with loss at 8p22 observed more frequently than at 8p21 (32). On the other hand, Aldaz et. al. found loss on 8p in only one of 15 informative samples of DCIS (7%) (33). At the time of our progress report last year we had assayed for LOH using 8 markers on 8p. Of 55 informative samples, LOH was found for at least one 8p marker in 15 tumors (27.3%) (34).

Because of the multiple putative tumor suppressor loci which exhibit LOH in invasive breast cancer, it is not clear which loci are involved in oncogenesis and which are lost randomly due to the instability conferred by the malignant state of the genome.

Allelotyping involves the comprehensive screen of the genome for LOH in a particular cancer. Generally an initial screen will involve assay with at least one marker from each non-acrocentric chromosomal arm. Thereby the average or baseline level of LOH can be determined. This may vary from 5 to 20% depending on the type of cancer. A significant level of LOH, indicating the site of possible tumor suppressor genes involved in oncogenesis, can be ascertained once the background level is known. Regions which show significant LOH can then be analyzed with additional markers to refine the smallest deleted region which may contain the tumor suppressor gene. The analysis of tumors with a number of markers also permits calculation of the fractional allelic loss (FAL) for each tumor. This has been defined as the total number of chromosomal arms which show LOH divided by the total number of informative arms for that tumor (35,36). FAL has been correlated with patient outcome in colon cancer (35), and may correlate with clinical information in other tumor types.

Fewer reports exist on the molecular changes in DCIS than can be found pertaining to invasive breast cancer. Davidoff et. al. (37) studied 6 examples of synchronous DCIS and invasive cancer for expression of p53 and found the same levels of protein expression in each tissue type. Expression of the oncogenes c-erbB-2 and c-myc is also consistent between coexisting pre-invasive and invasive breast cancer (38,39). Zhuang et. al. studied allelic loss for two loci on 11q13 (INT2 and PYGM). They found that for every case of DCIS which showed LOH (N=15), loss of the same allele was seen in the corresponding invasive tumor (40). O'Connell et. al. (41) studied four loci [TPO (2pter), D4S192 (4q25-34), D16S265 (16q21) and D17S579 (17q21)] and found that 8 of 10 cases of DCIS shared LOH patterns with more advanced lesions for at least one of the 4 loci.

During the first year of this project we completed the allelotyping of DCIS. Ours was the first laboratory to allelotype DCIS. Our findings were as follows: A total of 61 samples of DCIS were assayed. The average number of informative tumors examined for each marker was 19 (range 8 to 48). The median fractional allelic loss (FAL) was 0.037. The highest % of LOH was shown for loci on 8p (18.7%), 13q (18%), 16q (28.6%), 17p (37.5%) and 17q (15.9%). LOH on 18q was found in 10.7% of informative tumors. FAL was associated with LOH on 17p, with high nuclear grade and with the comedo subtype of DCIS. LOH on 17p correlated with LOH on 17q and on 13q. Additional markers were employed for 16q and 17p to determine the smallest common region of deletion and maps of 17p and 16q were generated (42-44). Aldaz et. al. also studied allelic loss in a total of 23 examples of DCIS. they found the most frequent sites of loss to be on chromosomes 7p, 16q, 17p and 17q (33).

To study genetic changes and the evolution of breast cancer we have assayed for loss of heterozygosity (LOH) in twelve sets of synchronous carcinoma *in situ* (CIS) and invasive cancer, compared to normal control DNA. Microsatellite markers were used which map to each non-acrocentric autosomal arm. Eight tumor sets demonstrated LOH of the same allele in both concurrent invasive cancer and DCIS, for a total of eighteen chromosomal loci. Three of nine tumor sets showed LOH on 11p. In two of these sets LOH was seen on 11p

only in the invasive tumor, not the corresponding CIS. One of these tumors also exhibited allelic loss in the invasive tumor for 4 loci, all of which were retained in the non-invasive tumor. For two tumor sets LOH was mirrored in matched DCIS, invasive tumor and lymph node metastasis. The maintenance of LOH for certain loci throughout the stages of breast cancer suggests clonality of the cancer cells. Tumor suppressor loci on 11p may be involved in the invasive phenotype (45).

During the second year of the project we have concentrated our efforts on the refinement of the area of loss on 8p. Simultaneously with that study we have generated a fine structure linkage map of 8p. Genetic mapping efforts indicated that the deleted region (between markers D8S520 and D8S265) spanned an interval of 1.4 cM. During the previous project year we have focused on the completion of physical (radiation hybrid) maps and construction of a clone contig for the region using yeast artificial chromosome (YAC) and bacterial artificial chromosomes (BAC). We have also identified and sequenced four apparently transcribed sequences that originate from the region of interest. Our current plans are to now focus on gene identification working mainly from the cloned DNA that spans the region.

#### **D. Purpose of the Present Work**

##### Revised Statement of Work:

We have accomplished Task 1, The identification and characterization of the extent of chromosomal deletions in DCIS (Months 1-12). As described in the previous progress report we have focused our efforts on Task 4: Cloning a tumor suppressor gene involved in breast cancer (Months 24-48).

Task 2, The study of chromosomal deletions in hyperproliferative breast conditions. (Months 12-24) and Task 3, The study of chromosomal deletions in the progression of DCIS to invasive and metastatic phenotypes (Months 1-36) will be taken up after Task 4 has been accomplished, time and effort permitting.

#### **E. Methods of Approach**

##### a) Accumulation of specimens.

Collaborations have been established with pathologists in St. Louis area hospitals. Archival paraffin embedded material is collected from several hospitals in St. Louis (Barnes-Jewish, Deaconess, St. Louis University, St. Luke's Hospital and the Outpatient Surgery Center). Either matched archival normal lymph node DNA or leukocyte DNA is used as control. When it is necessary to draw blood for normal control, informed consent is obtained following Institutional Review Board approval. A total of 89 examples of DCIS have been accumulated and assayed for LOH with various markers.

##### b) Microdissection.

For LOH analysis it is necessary to have a relatively pure tumor sample with little if any contaminating normal stroma. We have been using a microdissection technique to enrich for tumor cells in which an unstained 20 micron thick section from a particular block is overlaid on a stained 5 micron thick section. Landmarks such as blood vessels are aligned and the tumor dissected from the unstained section using a scalpel blade.

##### c) DNA extraction and LOH analysis.

Following separation of tumor and normal tissue DNA is extracted by digestion with proteinase K, purified with phenol/ chloroform and precipitated with alcohol. DNA is quantified with a fluorimeter. For assay of LOH we have used a panel of highly polymorphic microsatellite markers. Polymerase chain reaction (PCR) is performed in the tumor/normal pairs and the products separated on acrylamide denaturing gels. Reactions have been optimized for 5 to 10 ng of template DNA in order to maximize the number of reactions possible with each tumor. On autoradiography, absence or greatly reduced intensity of one allele in the tumor compared to the heterozygous normal control indicates LOH.

Several samples contain insufficient tumor cells to permit the extraction technique described above. New methods have been developed in order to consistently amplify via PCR these low quantities of DNA. Following microdissection the tumor tissue is digested in a small volume (10-20 microliters) of lysis buffer containing proteinase K. After complete digestion has been determined, samples are phenol extracted once to destroy the proteinase K and chloroform extracted once to remove the phenol. Aliquots of this material are then used directly as a template for PCR amplification. A disadvantage of this method is that only a limited number (10-20) of reactions can be done, and therefore this technique would not be suitable for an allelotyping study.

#### d) Generation of genetic linkage maps.

Once a region of chromosomal deletion has been identified it can be narrowed down using a panel of closely linked markers which map to that area. Since new microsatellite markers are becoming available daily, they often do not appear on currently published maps. In order to determine the deletion map in the tumors, it is necessary to know the precise location of the markers being used. A fine structure map can be generated using genotypic data from a number of families made available through the Centre d'Etude Polymorphisme Humaine (CEPH). Having identified a small region of deletion (preferably no larger than 1cM) positional cloning techniques can be undertaken to clone the putative tumor suppressor gene contained within the region.

#### e) Radiation hybrid maps

G3 and GB4 are the two radiation hybrid mapping panels (Research Genetics, Inc. Huntsville, AL) we used to construct the RH placement map. The G3 panel, comprised of 83 RH clones from the whole human genome, was created at the Stanford Human Genome Center and is considered a medium resolution panel (i.e. 500 Kb resolution). The GB4 panel, comprised of 93 RH clones from the whole human genome, has lower resolution (i.e. 1000 Kb). It is a subset of the 199 clone panel developed by the laboratories of Peter Goodfellow and Jean Weissenbach.

STS markers are assayed by PCR amplification and sizing on agarose gels stained with EtBr. Each assay is performed twice, i.e. PCR products at the expected size must be observed on each of the duplicate gels in order to be scored as positive. Data generated from the GB4 panel are submitted to the Whitehead Institute Center for Genome Research (WICGR) Mapping Service Center. The program RHMAPPER at the Center is used to analyze all the submitted markers with their high-likelihood framework map and we are then forwarded a placement map with all the submitted markers including LOD score and the distance in cR between two highest-linked markers. The data generated from the G3 panel is submitted to Stanford RH server which subsequently returns the results of analysis with a list of the highest-linked mapped markers, the LOD score of the link, and the distance in cR between the submitted marker and the linked marker on the map. However, it only compares one submitted marker and the highest-linked marker at a time. In order to construct a map of higher resolution which is obtainable with the G3 panel, we used the program RHMAPPER version 1.0 from WICGR, the Stanford RH database and our own

G3 data for the 6 markers of interest. We used the RH database from Stanford to build a G3 panel placement map (framework), then we integrated our RH data from the six markers covering the deletion region with the framework map.

f) YAC, BAC, P1 clone contig construction

YACs available from the CEPH library that we maintain in our laboratory were streaked on YPD plates, and 10 colonies from each clone were tested by a "whole cell PCR" assay using the STS markers to identify the positive clones. For "whole cell PCR, a small amount of cells from an isolated colony are suspended in 5 ul of deionized water and the suspension is used directly as the template in a standard PCR reaction. The presence of other markers within the YACs are also assayed by PCR assays of STS markers. The standard ligation-mediated PCR method was used to develop new STSs from YACs.

We screened a BAC library (Research Genetics, Inc., Huntsville, AL) to identify BAC clones for contig construction. STS assays for relevant markers were tested using the 120 standard PCR screening reactions to survey the STS content of the entire library. As with YAC clone isolation, each identified BAC clone was then verified by "whole cell PCR" assay using 10 randomly selected colonies as candidates. After the positive BAC clones were verified, single BAC colonies were propagated in liquid medium, cells harvested and insert DNA prepared using the Plasmid Midi-Kit from Qiagen Inc. (Chatsworth, CA). Each BAC clone was partially sequenced from the insert ends using T7 and Sp6 primers. The sequence generated from ABI sequencing was analyzed for candidate PCR primer sequences using the program PRIMER 0.5 (Lincoln and Lander, MIT Center for Genome Research). STS assays were developed and then used as new entry points for chromosome walking.

**Body: Experimental Methods Used and Results Obtained.**

**Task 4: Cloning a tumor suppressor gene involved in breast cancer. Months 24-48.**

**RH mapping**

In order to efficiently clone and characterize a putative tumor suppressor gene involved in breast cancer we have constructed a radiation hybrid map for the 1.4 cM deletion interval between markers D8S520 and D8S265 within chromosome 8p22-23. This map provides an independent means of ordering the markers in this region and helps to verify the linkage map marker order, a necessary step prior to the construction of a clone contig and gene identification. The radiation hybrid map also identifies additional markers (probably not polymorphic) that can be used to construct a clone contig for the region.

Initially six microsatellite markers, D8S265, D8S520, D8S550, D8S1695, D8S1755 and D8S1759 were used to screen the Stanford G3 and Genebridge 4 RH panels. Each marker was typed twice for each panel. Data from the Stanford G3 panel and the program RHMAPPING version 1.0 (WICGR) were used to construct an RH placement map described in the previous progress report. During this past year we have constructed a refined radiation hybrid map (Fig. 1) for the deletion interval using the Stanford G3 panel with resolution of 500 kb. In addition to the six microsatellite markers one STS (PLL), two ESTs, and marker WI-6800 were placed on the map using the data generated from the G3 panel and the program RHMap (v3.0). The marker order shown in Fig 1 supported the order of markers we had used for clone contig construction except at the position of D8S1755. The distance of 42.3 cR, or approximately 1565 kb (assuming 37 kb per cR)

between the markers D8S265 and D8S520 confirmed the 1.4 cM deletion region from our genetic mapping result.

### **Construction of an integrated YAC/BAC contig**

During this project year we completed construction of an integrated YAC/BAC contig spanning the 1.4 cM deletion region, the subject of our ongoing study. The gap between the markers D8S520 and D8S550 described in the previous progress report (1997) has now been filled with cloned DNA resulting in a single continuous clone contig for the region spanning a total distance of 1730 Kb. The region that contains the putative TSG is ~1240 kb in length which is about 400 kb larger than our estimate given in the previous progress report. The procedures described below were used to accomplish the project goals.

#### **BAC library screening**

The initial screening of the human BAC library (Research Genetics, Inc. Huntsville, AL) was conducted with 7 markers, D8S520, D8S550, D8S1755, WI-6800, D8S265, D8S1759, and 4 ESTs (1- 4) anchored on the YAC contig (Fig 2). An average of 5 BAC clones per marker were identified and isolated using PCR assays. After the positive BAC clones were verified, the insert ends of each clone were sequenced with T7 and SP6 primers. The sequence generated from ABI sequencing was analyzed for candidate STSs using the program PRIMER (Lincoln and Lander, MIT Center for Genome Research). Each newly generated STS was verified as to chromosome origin by rodent/human hybrid panel mapping. Only the clones containing chromosome-8 specific STSs at both ends were used for contig construction.

From our initial screening, we were able to build a continuous contig from D8S550 to D8S1759, leaving a gap between the markers of D8S520 and D8S550. We then used the novel STS PLL, developed from YAC 770E9 (1997 report) for BAC library screening. Three chromosome-8-specific clones A1, A2, and A3 were identified and isolated for STS-content mapping. After overlap analysis, 3 STSs generated from T7 ends of the clones were mapped back to YAC 770E9 and the STS A1-T7 tested positive for clone C2 identified by the marker D8S520 which placed clones A1, A2, and A3 to the distal side of the contig and linked the marker PLL to the marker D8S520 with a possible deletion contained within our YAC clone 770E9.

#### **Chromosome walking**

To close the gap between D8S520 and D8S550, STSs developed from T7-end sequences of clones C1 and G1 were used for secondary BAC library screening. Three clones, D1, D2, and D3, and one clone, F1, were identified and isolated from STSs C1-T7 and STS G1-T7, respectively. Both insert ends of clones D1, D2, D3 and F1 were sequenced and 6 chromosome-8-specific STSs were generated. STSs, D1-SP6, D1-T7, D2-SP6, D2-T7, D3-SP6, and F1-T7 were mapped back onto the YAC/BAC contig using PCR assays. However STSs developed from D2-T7, and F1-SP6 did not test positive for existing clones on the contig. Thus, additional BAC clones were needed to connect the remaining gap between these two markers. From another BAC library screening experiment, 5 clones were identified and isolated using a PCR assay from the marker F1-SP6; 4 of them were found to be positive for the marker from D3-T7. Both insert ends of the newly identified clones, E1 and E2 were sequenced and 4 novel STSs, E1-T7, E1-SP6, E2T7, and E2-SP6 were developed and mapped back to the contig by overlap analysis. This completed construction of a continuous BAC clone-based contig of the region.

All of the 27 BAC clones used for contig construction were digested with restriction enzyme Not I from which estimates of the sizes of the clones could be made. This information enabled more precise determination of the size of the region spanned by the contig and of the region containing the putative TSG. The contig now stands at approximately 1730 kb with the deletion (TSG-containing) region between D8S520 and D8S265 at 1240 kb (i.e. based on the estimate of sizes of the BAC clones) (Fig. 2).

#### YAC library rescreening

In order to confirm the continuity of the BAC clone-based contig, a new YAC clone 770E9 from Research Genetics was streaked on YPD plate, and 10 colonies were tested using "whole cell PCR" assay with the marker D8S520. Positive colonies were then probed for STSs for the region between D8S520 and D8S550 and found to be positive for all the STSs tested that comprised our entire collection for this region.

#### TSG Candidate Identification

First, databases were searched for candidate genes at the region of chromosome 8p22-23. We verified by PCR assay the locations of four ESTs reported to place within this region. EST 1 identified by TIGR mapped to YAC 770E9. It tested positive for BAC clones A1, A2, and A3. One of the cDNAs (AA609519) identified by EST1 has been isolated and sequencing is in progress. EST 2 mapped to YACs 770E9, 915H4, 715C10, 737E5, and 729E12. It also tested positive for BAC clones J1 and J2 identified by D8S265 and EST 1. Two cDNA clones (T96924, 0.8 Kb and W67504, 1.6 Kb) were isolated and fully sequenced. The two sequences were compiled into one continuous sequence using clustalW alignment of MacVector program. This sequence contains (CA)<sub>22</sub> repeats which could be used to develop a microsatellite marker. BAC clones, J1 and J2 were also partially sequenced with a primer selected from the 3' end of cDNA T96924. The sequences from these two BAC clones contain a (CA)<sub>22</sub> repeat and matched the sequence from cDNA W67504. Both ESTs 3 and 4 mapped to YAC 809H8 and tested positive for BAC clone L2. EST 1, 2, and 4 appear to be unique sequence since they did not identify homologous sequence in the publicly available databases. EST 3 which is one of the ESTs identified by the marker SGC30677 is derived from a cDNA clone similar to human Farnesyl Diphosphate Farnesyltransferase.

In addition to the previously reported ESTs from the region, both end sequences generated from BAC clones were analyzed with dbEST and BlastN. Only the Sp6-end sequence from clone K1 identified a homologous sequence, i.e. Stratagene's fetal retina Homo sapiens cDNA clone (AA504989). About 1.5 Kb of this cDNA clone has been sequenced. Another cDNA clone (W68256, 0.6 Kb) identified by the marker WI6800 was isolated and fully sequenced.

#### Conclusions

Our radiation hybrid mapping efforts conducted during the previous project year have independently determined the order of genetic markers for the smallest region of deletion found from earlier work. The physical distance spanned by the critical markers appears to be 17 cR or approximately 510 Kb - 850 Kb (physical size estimate based on cR/kb estimates). The RH map also identified additional markers within the interval from which a clone contig construction project was initiated. YACs were identified from this region and one gap (between markers D8S520 and D8S550) was identified. Through a combination

of YAC and BAC screening, STS development from end clone sequences, and rescreening the YAC and BAC libraries, this past year we closed the gap between these critical markers. Not I restriction enzyme mapping enabled more direct measurements of the size of the critical region, which is now estimated to span 1240 kb. This distance is approximately 400 kb larger than previously estimated. There are several possible explanations for the apparent increase in size for the critical region. Our recent RH mapping work has utilized the RHMap program which is thought to give more reliable size estimates than the previously used RHMPPER program. In addition, we integrated more markers into our current RH map. Map expansion often occurs as more markers are added often due to data errors, analogous to map expansion caused by data errors in linkage mapping. Our recent estimates for the physical distance are based on size estimates of clones digested with Not I, which is more likely to be accurate than estimates of kb per centiRad (the only means of size estimate we were able to make last year). Even though the size of the critical region is somewhat larger than previously thought, this size (1240 kb) is still within the range that is feasible for gene identification by a small research team. Future studies will focus on DNA sequence analysis and exon trapping using the contig resource. New microsatellite markers isolated from the critical region will be used to further characterize the minimum deleted region in tumors. This will help us to prioritize sequencing of candidate genes together with analysis of the expression pattern of each gene.

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## Appendices

Figure 1. G3 RH placement map constructed by typing 7 markers and 3 ESTs within the deletion region.

Figure 2. Integrated YAC/BAC contig between the markers of D8S520 and D8S550.

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Marker	Distance (cR)
Telomere	
SHGC-1955	4.7
EST 1	4.8
PLL	4.9
D8S520	20.8
D8S550	16.2
WI-6800	0.0
EST 2	5.3
D8S265	20.6
D8S1755	17.6
D8S1759	0.0
D8S1695	11.6
SHGC-13122	
Centromere	

Figure 1: G3 RH placement map constructed by typing 7 markers and 3 ESTs covering the deletion region.

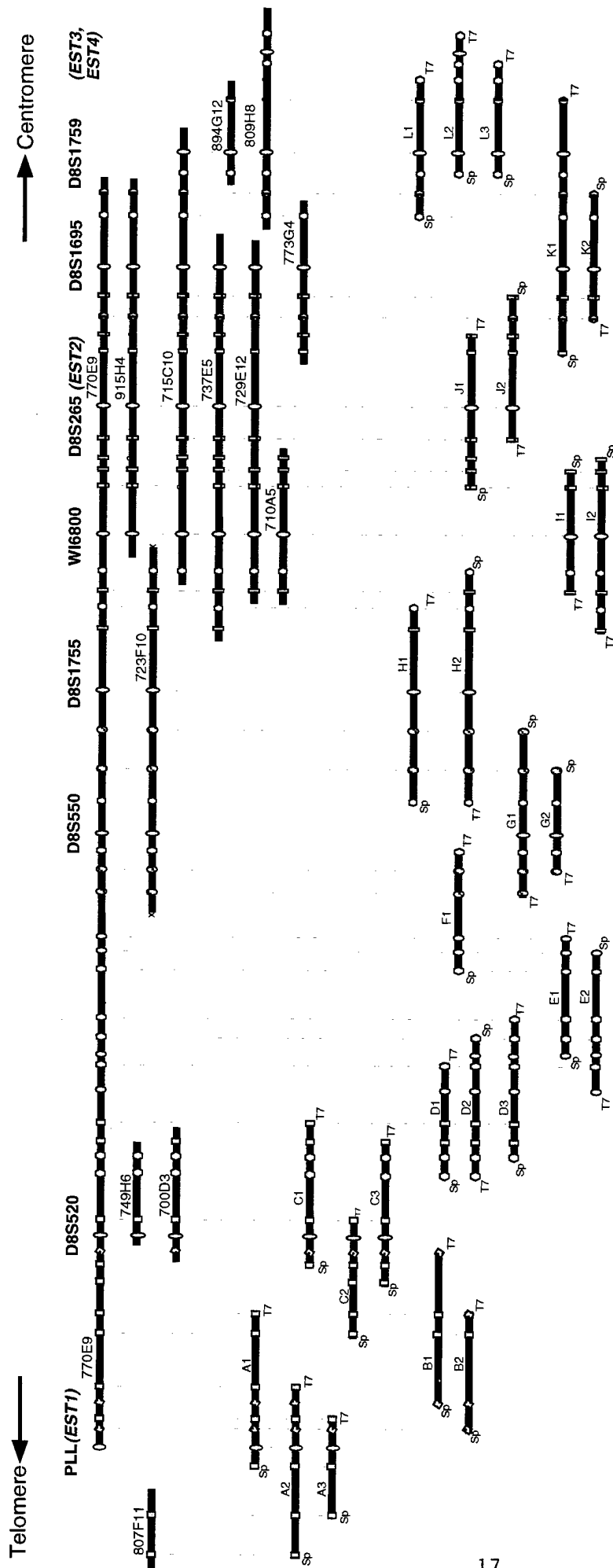


Figure 2: Integrated YAC/BAC contig on Chromosome 8p22-23 covering the 1.4 cM deletion region

—: YAC clones    —: BAC clones