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Estrogens are important regulators of growth and differentiation in the normal mammary gland and are also important in the development and progression of breast carcinoma. Estrogens regulate gene expression via estrogen receptor (ER) protein and, because approximately two-thirds of all breast cancers are ER+ at the time of diagnosis, the expression of the receptor has important implications for their biology and therapy.

ER binds to the estrogen response element (ERE) found in the promoters of estrogen-regulated genes and activates their transcription. Several cofactors (coactivators and corepressors) have been identified that are of importance in regulating the ER interaction with the basal transcription machinery.

Herein, we will address the role of specific cofactors in ER action and the rules that govern the specific recruitment of these proteins to specific promoters.

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Introduction

Estrogens are important regulators of growth and differentiation in the normal mammary gland and during the development and progression of breast carcinoma (**1**). Estrogens regulate gene expression through estrogen receptor (ER) protein and the estrogen response elements (ERE) found in promoters of estrogen-regulated genes (**2**). This regulation occurs through cofactor proteins recruited by ER to the promoter. Those proteins act mediating the indirect interaction of ER to the basal transcription machinery (**3**). The aim of this grant is to study the role of several cofactors (coactivators and corepressors) in breast development and receptor-dependent tumorigenesis. Thus, three different approaches have been designed (see *Original Proposal* and *First Annual Report* for details).

The first is related to the protein p/CIP/AIB1/ACTR/RAC3/TRAM-1. This is a coactivator identified by its interaction with nuclear receptors in an agonist-dependent manner (**4, 5**). It is highly amplified in 10% of primary breast cancers and overexpressed in 64% of breast cancers examined (**6**). On the other hand, NCoR is a corepressor identified by its interaction with nuclear receptors in an antagonist-dependent manner (**7, 8**). The susceptibility to breast cancer will be analyzed in transgenic mouse lines overexpressing p/CIP or NCoR in breast tissue, and a genetic background of expression or deletion of NCoR and p/CIP proteins.

The second approach is related with the coactivator β-catenin. Diverse evidence suggests a role for Wnt signalling pathway in mammary gland development and tumorigenesis (**9**). One pivotal element of this pathway is the coactivator β-catenin, which is an oncogenic protein responsible for multiple cancers (**10, 11, 12**). It translocates to the nucleus and activates the gene expression of specific genes under Wnt signalling induction. Nevertheless, the mechanism of this activation and whether estrogen and Wnt signalling pathways crosstalk through β-catenin/ER interaction are not well understood. Purification and identification of β-catenin nuclear multiprotein complexes will address both questions.

The third approach is related to the deciphering of the rules that determine the recruitment of p/CIP, NCoR, β-catenin and other coactivators and corepressors to specific promoters. To date, there are a large number of cofactors identified (**3**). Transcription factors, as ER, recruit those molecules in a promoter-specific, ligand- and time-dependent manner, but not many rules are known for this recruitment. The use of an amazingly powerful methodology as is ChIP-chip technology (**13, 14, 15**) will help us to define those rules by studying the *in vivo*

genome-wide recruitment of different cofactors under different conditions at different times. To achieve this, the mouse promoter array will be built and will allow me to address those questions proposed in this grant using mouse models.

The second year of this grant has been extremely important to develop the tools that finally have to be crucial to finish the projects during the third year. Some of these tools were described in detail in the *Original Proposal*. Additions explained in the *First Annual Report* are now added to the updated *Statement of Work* enclosed with this report. Those modifications positively effect to the original goals of this grant in the understanding of the role of different cofactors (coactivators and corepressors) in breast development and receptor-dependent tumorigenesis.

Body

The results obtained this year are presented in three independent sections, according to the new *Statement of Work*:

(A) The colony of mice that overexpressed p/CIP specifically in mammary gland was appropriately expanded during this year. Interestingly, preliminary data show that those mice present increased susceptibility to breast tumor formation as was evident by simple histological analysis. After this, the response to pregnancy, lactating and involuting will be analyzed in the next months.

In the other hand, it has not yet been possible to generate phenotypes in transgenic mouse lines overexpressing NCoR. Unexpected problems in infection of these lines have been detected, necessitating repeating experiments.

B) The purification of nuclear multiprotein complexes of β-catenin by using TAP-methodology follows the protocol described in the *First Annual Report* (**Figure 1.A**). A mutated form of β-catenin that constitutively localize inside the nucleus (β-catenin$_c$) was cloned in the expression plasmid pcDNA3-TAP (generous gift from Dr. R. Gherzi). That plasmid (pcDNA3-β-catenin$_c$-TAP) was transiently transfected in 293 cells to test its ability to express the fusion protein β-catenin$_c$-TAP. A new β-catenin form of expected size was detected by Western blot (**Figure 1.B, upper panel**). Nuclear and cytoplasmic fractionation was performed to determine the ability of the new form to localize, at least partially, inside the nucleus (**Figure 1.B, lower panel**). The *in vivo* functionality of the

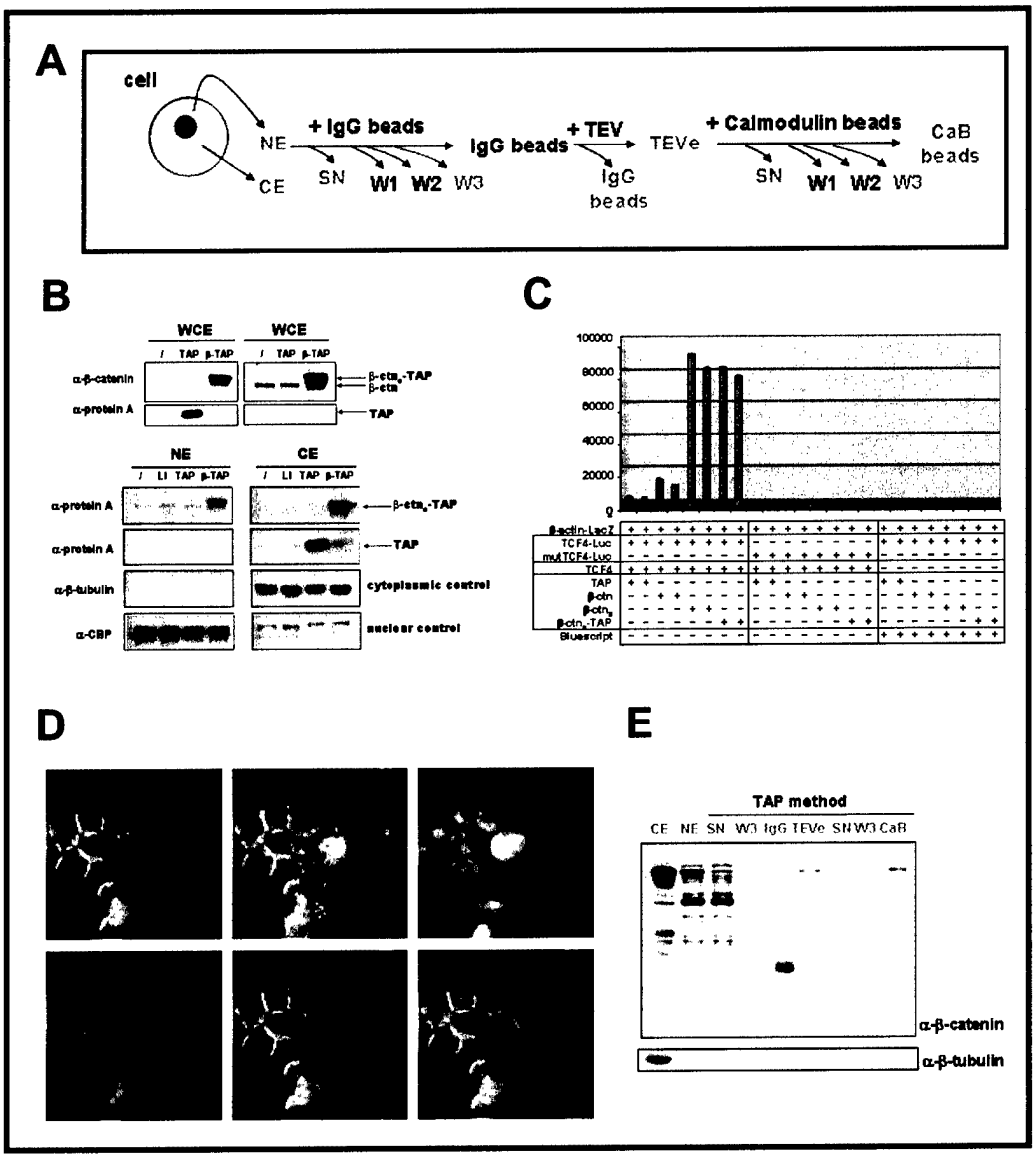

Figure 1.- A) The TAP protocol for purification of multiprotein complexes. Cells are fractionated to obtain nuclear (**NE**) and cytoplasmic (**CE**) extracts. **First step of purification**: NE is incubated with immunoglobulin beads (**IgG beads**). Not bound material (**SN**), first wash (**W1**), second wash (**W2**), third wash (**W3**). **Second step of purification**: Bound proteins to IgG beads are eluted by TEV protease cleavage. **Third step of purification**: Eluted material from the second step of purification (**TEVe**) is incubated with calmodulin beads. Finally, multiprotein complexes are retained by calmodulin beads (**CaB beads**). **B) Expression of β-catenin$_c$-TAP in 293 cells.** Whole cellular extracts (**WCE**) and nuclear and cytoplasmic extracts are analyzed by Western blot using antibodies against β-catenin, protein A, β-tubulin and CBP. Extracts from wild type (*I*), TAP-transfected (**TAP**) and β-catenin$_c$-TAP-transfected (**β-TAP**) 293 cells were obtained. **C) Analysis of the β-catenin$_c$-TAP functionality by reporter gene assay.** Expression plasmids for TAP, wild type β-catenin, β-catenin$_c$ and β-catenin$_c$-TAP were cotransfected in 293 cells with the expression plasmid for the transcription factor TCF4, and the reporter plasmid with the luciferase-reporter gene under the control of a TCF4-responsive promoter (**TCF4-luc**) or a mutated TCF4-non-responsive promoter (**mutTCF4**). **D) Immunostaining of β-catenin$_c$-TAP stably transfected 293 cells.** Detection: an antibody against protein A (**Prot A**) for β-catenin$_c$-TAP detection, phalloidine (**Actin**) for cytoplasmic detection and DAPI for nuclear detection were used. **E) TAP protocol with the 293-derived β-catenin$_c$-TAP stable cell line.** A Western blot using samples from different steps of the TAP protocol (see A) is shown. Antibodies against β-catenin and β-tubulin were used. β-catenin$_c$-TAP is retained by both columns.

new protein was confirmed by gene reporter assays were β-catenin$_c$-TAP was able to activate the transcription of a luciferase reporter gene in a TCF4-responsive promoter dependent manner (**Figure 1.C**). Cotransfections were done in 293 cells using a plasmid that express the transcription factor TCF4 and a plasmid that has the reporter gene under the control of a promoter with a wild type-TCF-responsive sequence or a mutated-TCF-non-responsive sequence (pHR-TCF4, pGL3-OT and pGL3-OF, respectively, generous gift from Dr. B. Vogelstein and Dr. K.W. Kinzler). These results indicate that it is possible to express nuclear and functional β-catenin$_c$-TAP using the plasmid pcDNA3-β-catenin$_c$-TAP.

The high expression levels of the fusion protein and the necessity of a relatively large amount of material made necessary the generation of a β-catenin$_c$-TAP stably expressing cell line. Four different cell lines were transfected with the plasmid pcDNA3-β-catenin$_c$-TAP: human MCF-7, LNCaP and 293, and mouse *α*-T3 cell lines (the *Claim of Exemption Form* for the use of these cell lines is enclosed with this report). After clone selection growing in selective media, different clones were obtained. All of them integrated the plasmid, but not all of them expressed the fusion protein β-catenin$_c$-TAP. Seventeen MCF-7 derived clones were tested and none of them expressed β-catenin-TAP. The same result was observed when *α*-T3 derived clones were tested. One explanation for this lack of expression is that the expression of β-catenin$_c$-TAP could be toxic for both cell lines, and although some clones were stably transfected, they probably were selected because their low levels of protein expression. Nevertheless, some 293 and LNCaP-derived clones were successfully obtained that expressed β-catenin$_c$-TAP. Those clones expressed the protein in a similar level than the endogenous β-catenin protein. As observed in transiently transfection (**Figure 1.B, lower panel**), β-catenin$_c$-TAP was soluble and constitutively localized inside the nucleus (data not shown). However, a large amount of protein was also insoluble. Immunostaning experiments surprisingly showed that most of the fusion protein was bound to the cellular membrane in a similar distribution pattern of the wild-type β-catenin (**Figure 1.D**). These results indicate that although was not yet successful in generation of a β-catenin$_c$-TAP expressing cell line derived from the human MCF7, LNCaP and 293-derived clones were available. Those two cell lines, while not breast cancer models, their use permit the purification and identification of stable β-catenin nuclear partners, and can be assessed in their possible relation with ER and the estrogen signalling pathways using wild-type MCF7 cells.

Finally, the TAP-purification protocol was tested with one of the 293-derived clones (see *First Annual Report* for details about the protocol). Nuclear extracts

from this clone were obtained and sequentially loaded in IgG- and calmodulin-columns (**Figure 1.A**). Samples from the different steps of purification were tested by Western blot (**Figure 1.E**). As it is shown, β-catenin$_c$-TAP was specifically retained by both columns. These results indicate that this clone is valid to perform the β-catenin$_c$-TAP complex purification. The next step will be to produce large amounts of nuclear extract to obtain enough material to identify by MALDI-spectrometry the β-catenin$_c$-TAP associated proteins.

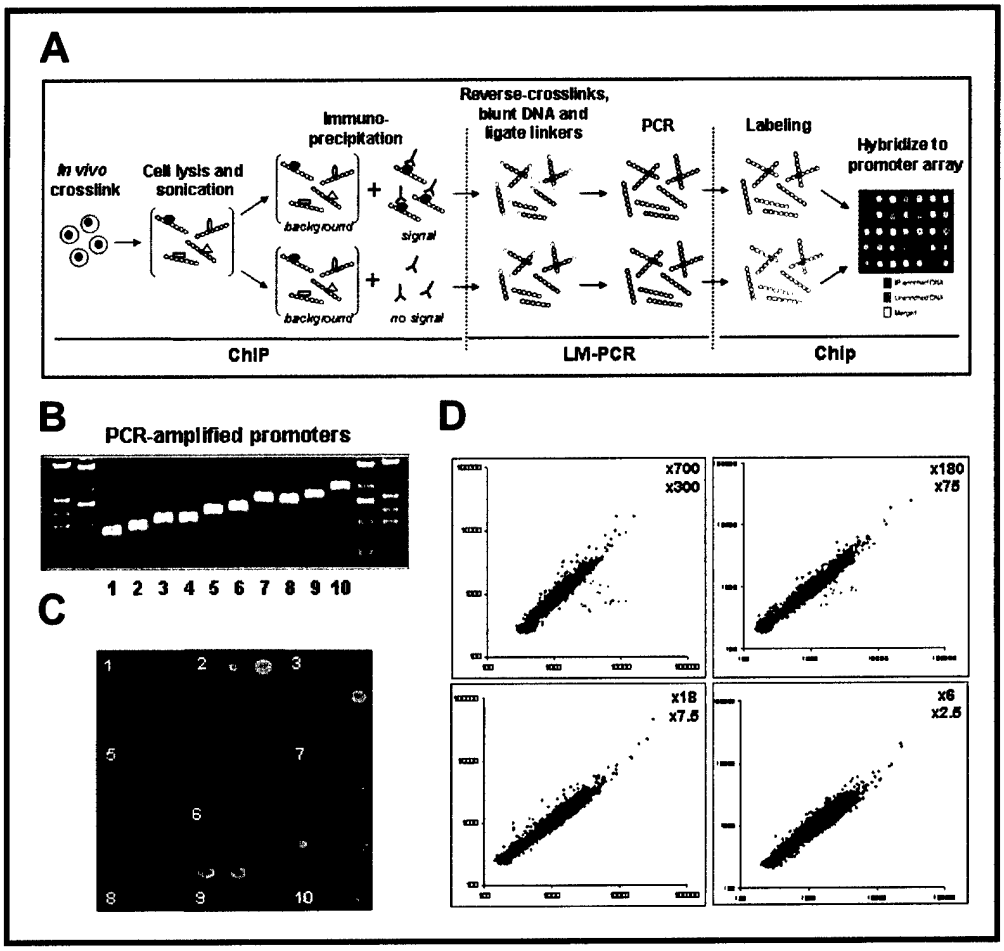

Figure 2.- A) ChIP-chip methodology. This is a combination of a chromatin immunoprecipitation experiment (**ChIP**) and a chip microarray experiment (**chip**), using ligation-mediated PCR (LM-PCR) between both to amplify the signal. **B) PCR-amplified promoters used to test the array.** Ten different promoters were PCR-amplified, purified and pooled to test the sensitivity of the mouse promoter array. **C) Mouse promoter array hybridized with the pooled sample of promoters. D) Analysis of the sensitivity using different fold excess of the pooled promoters.** X values are Cy5 intensities. Y values are Cy3 intensities. Orange dots represent the PCR-amplified promoters. Blue dots represent the non-PCR-amplified promoters of the array.

C) ChIP-chip methodology (**Figure 2.A**) has been successfully used in yeast and human systems, but never before in mouse. The work with this last system has several advantages, as the possibility to study the effect of the deletion or overexpression of a specific protein in breast development. For that reason we thought the mouse version of the promoter array could be very useful for the purposes of this grant. The design and building of the first version of the mouse promoter array has advanced significantly this last year. That is the product of the collaboration between Dr. M.G. Rosenfeld and C. Glass labs, and I am participating in this exciting project. As a first pilot, around one thousand genes were selected for our purposes. These comprised genes that are interesting for the object of this grant. Those are more than 200 hundred genes implicated in regulation of the cell cycle, with an evident interest in cancer, and more than 300 hundred genes implicated in regulation of the gene transcription, mostly cofactors (coactivators and corepressors). Dr. J. Lozach (Dr. Glass lab) obtained the DNA sequence of the whole list of promoters and designed primers for the amplification of the region between +200 and -800 bp from the start site of the promoter. Several fragments were amplified when alternative promoters were detected. The PCR-amplifications were performed by BIOGEM (Dr. G. Hardiman-UCSD), and they took care also of the sequencing step. The identity of each PCR-amplified fragment as a specific promoter was confirmed by using BLAST program and the data from the sequencing. Once all the fragments were confirmed, BIOGEM printed them in spots on glass slides.

To test the mouse promoter array, ten promoters were randomly selected from the cofactors list. They were between 400 bp and 1000 bp in size (**Figure 2.B**). After PCR-amplification and DNA purification, all of them were accurately quantified and mixed. Serial dilutions of the pooled sample and a genomic DNA sample were tested and compared by PCR (data not shown). The necessary amounts to obtain the same material after the PCR-amplifications were taken as a reference. An excess of x700-300, x180-75, x18-7.5 and x6-2.5 (depending of the promoter) of the pooled sample was added to 200ng of mouse genomic DNA, and the mix was labelled by random priming with the dye Cy5. The same amount of genomic DNA without the excess of pooled promoters was labelled with the dye Cy3. Equivalent amounts of both labelled DNA samples were mixed and hybridized with the mouse promoter array.

Nine of the ten pooled promoters were detected using an excess of x700-300 pooled promoters (**Figure 2.C**). Decreasing the fold excess to x180-75, two of the signals were missed and, finally, working with x18-7.5 and x6-2.5 all of them were fully disappeared (**Figure 2.D**). Those results show that the mouse promoter array is perfectly working, but the sensitivity seems to be lower than the initially expected. However, promising preliminary data seems to show an

increase of sensitivity to x18-7.5 using different conditions (data not shown). That would make possible the use of this array with samples obtained from chromatin immunoprecipitations, were the expected fold of enrichment is close to that last fold.

Key research accomplishments

- Colony expansion of the p/CIP-overexpressing transgenic mouse line (**Section A**).
- Generation and characterization of a stable cell line that express a functional β-catenin-TAP protein suitable for TAP purification, as it has been demonstrated in **Section B**.
- Generation and testing of the first mouse promoter array with more than one thousand promoters. The array works with test samples and is ready for the use with real samples, as it is shown in **Section C**.

Conclusions

The second year of support under the DoD Breast Cancer Research Award, I have made significant progress developing the tools I need to achieve the final aims of this grant. Unfortunately, several unexpected problems were detected in the generation of the NCoR-transgenic mice lines, as described in this report. Proposed additions described in the *First Annual Report* are now added to a new *Statement of Work* enclosed with this report (**Appendices**). A *Claim of Exemption Form* for the use of cell lines is also enclosed with this report (**Appendices**).

These developed tools have to be very useful for the study of the role of several cofactors (coactivators and corepressors) in breast development and receptor-dependent tumorigenesis. Specifically, they will be directed to determining the role of the coactivator p/CIP in breast cancer and development (**Section A**), determining if there is a nuclear connection between estrogen and Wnt signalling pathways through the coactivator β-catenin (**Section B**), and determining the rules that determine the specific recruitment of a cofactor, as p/CIP, NCoR, β-catenin, and others, on a specific promoter by a specific transcription factor, as ER (**Section C**).

Therefore, this year has been very positive for the goals of this grant and also extremely useful for my training in several new methodologies that I want to introduce in the field of breast cancer and development.

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15. Claim of Exemption Form

PROTOCOL TITLE:
Coactivators and corepressors in breast development and receptor-dependent tumorigenesis
PRINCIPAL INVESTIGATOR'S NAME: / PROPOSAL NO:
Ivan Garcia Bassets, PhD / BC000882
INSTITUTION:
University of California, San Diego (UCSD)

	Yes	No
1. Will existing or archived data, documents, medical records, or database records be used?	☐	X
2. Will biological specimens (e.g., cells, tissues, blood) be used?	☐	X

3. Indicate below the sources of existing or archived data or biological specimens or cell lines (e.g., cell lines purchased from ATCC).

Cell lines purchased from ATCC

	Yes	No
4. Will the donors of the original biological specimens be able to be identified, directly or indirectly, through identifiers linked to the donor?	☐	X
5. Will data be recorded in writing?	☐	X
6. Will data be recorded by audiotape?	☐	X
7. Will data be recorded by videotape?	☐	X
8. If survey instruments are used, will sensitive or private topics be explored?	☐	X
9. Will subjects be identifiable either by name or through demographic data?	☐	X

If the answer to any question 4-9 is yes, describe on a separate sheet of paper how the confidentiality of a subject's identity will be maintained. Also describe plans for maintaining or destroying identifying links to subjects after the protocol has been completed.

Principal Investigator's Signature

10/02/02
Date

Statement of Work

Task I. To construct breast specific p/CIP-overexpressing transgenic mouse lines in a context of wild type and p/CIP-deleted genes, and to compare growth, development and function of the mammary gland on those mice.

(a) Generation of transgene targeting contructs under the control of WAP (whey acidic protein) promoter (Months 0-12).
(b) Microinjection of these constructs into wild type and p/CIP-deleted embryos (Months 6-12) and expansion of the colonies of transgenic mice (Months 8-24).
(c) Confirmation of the transgene overexpression (Months 12-20) and breeding (Months 16-26).
(d) Analysis of immature and mature virgin phenotypes from serial transplanted hosts (Months 16-28) and analysis of pregnant, lactating and involuting transgenic lines (Months 16-36).
(e) Serial transplantation of mammary outgrowths from first generation of hosts (Months 18-36)
(f) Comparison of tumor loading and susceptibility of these lines (Months 25-36).

Task II. To purify and to identify nuclear β-catenin stable partners, and to determine if there is a nuclear connection between ER and Wnt signalling pathways.

(a) Generation of constructs that express the fusion protein β-catenin-TAP (Months 8-12).
(b) Confirmation of the expression and testing the functionality of the expressed fusion protein in transiently transfected cell lines (Months 12-18).
(c) Generation of stable cell lines that express the fusion protein and selection of the appropriate clones (Months 18-24).
(d) Expansion of the selected clones and purification of complexes by using the TAP methodology (Months 24-30)
(e) Identification of the purified proteins by MALDI-spectrometry methodology (Months 24-30).
(f) Analysis of the interaction of the identified proteins with β-catenin and ER proteins (Months 28-36).

Task III. To determine the rules that define the recruitment of a specific cofactor to a specific promoter.

(a) Generation of the mouse promoter array: selection of the genes, determination of the promoter regions, oligonucleotide design, promoter PCR-amplification, sequencing of the amplified fragments and printing of the spots on the array (Months 8-22).
(b) Determination of the appropriate conditions for the use of the mouse promoter array: sample, labelling and hybridization conditions (Months 22-30).

(c) Hybridization of the mouse promoter array with chromatin immunoprecipitated samples obtained using antibodies against different cofactors. Samples from different cellular conditions will we tested to determine the rules that determine the cofactor recruitment on the promoters (Months 28-36).