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TITLE: The Role of the Rab Coupling Protein in ErbB2-Driven Mammary Tumorigenesis and Metastasis

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Fort Detrick, Maryland 21702-5012

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### Title and Subtitle
The Role of the Rab Coupling Protein in ErbB2-Driven Mammary Tumorigenesis and Metastasis

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### Sponsor/Monitor's Name and Address
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

### ABSTRACT
During the Year-1 we characterized the role of RCP in ErbB2-driven breast cancer model. To directly evaluate the RCP in ErbB2 mammary tumorigenesis, we have derived transgenic mice that inductively express RCP in the mammary epithelium and interbreed these mice with separate strain expressing an MMTV activated ErbB2 transgene. Induction of RCP expression in the ErbB2-driven model results in a tumor onset delay in addition to a drastic defect in metastasis formation. Furthermore, elevated RCP expression promotes a significant decrease in cell proliferation that is associated with a Senescence Associated to Heterochromatin Foci cellular senescence phenotype. In addition, using *in vitro* 3D-cell culture model of ErbB2 positive breast cancer, we showed that RCP is essential to the maintenance of adherens junctions by regulating E-cadherin endocytosis. Conversely depletion of RCP in ErbB2 positive cell lines leads to a significant increase in the number of lung metastases. Consistent with the importance of RCP as negative regulator of ErbB2 positive breast cancer progression, gene expression profile analysis of tumor banks of human breast tumors showed that the expression of RCP is inversely correlated with ErbB2 levels. Overall, ether, these observations argue that RCP acts as a tumor suppressor in ErbB2-driven breast cancer progression.

### Subject Terms
Breast Cancer, Oncogene-Induced Tumorigenesis, Metastasis Formation, Receptor Tyrosine Kinase, Her/ErbB2 signaling, Rab Coupling Protein/Rab11FIP1/RCP, Epithelial Mesenchymal Transition, Cell junctions, Cell Proliferation, Senescence.
Table of Contents

1. Introduction ................................................................. 5
2. Keywords ....................................................................... 5
3. Accomplishments ............................................................ 5
4. Impact ........................................................................... 10
   5. Changes/Problems ....................................................... 11
   6. Products ...................................................................... 11
7. Participants & Other Collaborating Organizations ............. 13
8. Special Reporting Requirements ........................................ 15
9. Appendices .................................................................... 15
INTRODUCTION

Amplification and elevated expression of Rab Coupling Protein (Rab11FIP1/RCP) has been noted in 10-25% of primary breast cancers and was further correlated with high metastatic grade and poor clinical outcome. Nevertheless, the role of RCP in ErbB2-positive breast cancer remains elusive. In this study, we report that RCP plays a critical role in modulating ErbB2 dependent mammary tumor progresssion and metastasis. To directly evaluate the RCP in ErbB2 mammary tumorigenesis, we have derived transgenic mice that inducibly express RCP in the mammary epithelium and interbreed these mice with separate strain expressing an MMTV activated ErbB2 transgene. Induction of RCP expression in the ErbB2-driven model results in a tumor onset delay in addition to a drastic defect in metastasis formation. Furthermore, elevated RCP expression promotes a significant decrease in cell proliferation that is associated with a Senescence Associated to Heterochromatin Foci (SAHF) cellular senescence phenotype. In addition, using in vitro 3D-cell culture model of ErbB2 positive breast cancer, we showed that RCP is essential to the maintenance of adherens junctions by regulating E-cadherin endocytosis. Conversely depletion of RCP in ErbB2 positive cell lines leads to a significant increase in the number of lung metastases. Consistent with the importance of RCP as negative regulator of ErbB2 positive breast cancer progression, gene expression profile analysis of tumor banks of human breast tumors showed that the expression of RCP is inversely correlated with ErbB2 levels. Taken together, these observations argue that RCP acts as a tumor suppressor in ErbB2-driven breast cancer progression.

KEYWORDS

Breast Cancer, Oncogene-Induced Tumorigenesis, Metastasis Formation, Receptor Tyrosine Kinase, Her/ErbB2 signaling, Rab Coupling Protein/Rab11FIP1/RCP, Epithelial Mesenchymal Transition, Cell junctions, Cell Proliferation, Senescence.

ACCOMPLISHMENTS

The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

3.1 Major goals of the project

To summarize the main goal of this research proposal is to understand to functional role of RCP in ErbB2 amplified breast cancer model. To accomplish this, we examined the role of RCP using two scientific approaches routinely used to validate new gene target in breast cancer research such as in vitro cell model and in vivo mouse model. This goal is subdivided into two main objectives as below.

SOW-Objective 1. To elucidate the role of RCP in ErbB2 endocytosis as well as in Histone-3 methylation to control oncogenic activity of ErbB2 receptor.

SOW-Objective 2. Characterize mammary tumour progression as well as metastasis formation in RCP-proficient and RCP-deficient mouse model.

In this following section only SOW-Tasks related to Year-1 are discuss and all Tasks linked to Year-2 are indicated in the appropriate section.
SOW-Task 1. Attain ethical approval from ACURO for the use of mice (Months 2-3): Completion: 100%.

In order to respect the ethical approval from ACURO regarding the use of the mice, our Molecular Oncology Group, currently holds an approved Animal Use Protocol # 2008-5518 with McGill University following the high standards established by the Canadian Council on Animal Care.

SOW-Objective-1.

SOW-Task 2. Determine the specific endocytic route of ErbB2 in RCP-deficient and -proficient primary ErbB2 tumor cells (Months 1-6): Completion: 50%.

2a. Infect SKBR3 and BT474 cell lines with RCP-GFP or lentiviral-RCP-RNAi (shRNA) construct. Cells pre-treated or not with Clathrin or lipid raft inhibitors and assess internalization of ErbB2 using cold/warm temperature switches under confocal and super-resolution localization microscopy and cell-surface receptor biotinylation assays.

During Year-1, I established SKBR3 stable expressed cell lines overexpressing RCP-GFP and depleted in RCP using shRNA coupled to lentiviral infection. We have tried to optimize the use of commercially available Clathrin and lipid raft inhibitors. Using different dose concentration we detect no significant change in terms of ErbB2 receptor internalization. For monitoring reason, we have preferred to use only the temperature switch assay than classic biotinylation approach. Our results present no interesting feature. Thus, as presented in our preliminary data, using the ErbB2/neuNT TM15 cells, the overexpression of RCP led into stabilization of the receptor at cell surface (see narrative section in official application). In the meantime, in all TM15, SKBR3 and BT474 cell lines we noticed that the adherent junction cell marker refered hereafter as E-cadherin constitutes a potent target in ErbB2-driven model instead of the ErbB2 receptor itself.

2b. Infect TM15 cell clones with His-RCP (instead of RCP-GFP for protein detection reason) or RCP-shRNA and similar approach as in Obj.1 Task-2a. Tracking of distribution of ErbB2 using endosomal markers in addition to lysosome and multivesicular bodies staining. Confirm modulation of ErbB2 oncogenic activity by assessed indicated RCP-ErbB2 cell models to proliferation assay, migration and invasion assay as well as biochemical studies.

Using both ectopic expression and RNA interference approaches, we derived pooled stable TM15 cell line that exhibited either a 2 fold increase in RCP levels or produced a significant endogenous RCP knockdown (Fig. 1A) as demonstrated by a 96% reduction in the levels of RCP (Fig. 1B). Consistent with the ability of RCP to modulate the proliferative status of ErbB2 tumor cells, ectopic expression of RCP reduced the proliferative capacity whereas reduction in RCP increased the proliferative potential relative to the parental cell line (Fig. 1C). Interestingly, the overexpression of RCP show significant change in terms of cell migration fate. In contrast, the depletion of RCP showed a dramatic increase of cell migration rate (Fig 2). However, we push forward the idea that RCP can regulates cell proliferation fate of ErbB2 cells through the cell junction modulation involving stabilization at cell surface of ZONAB a well-characterized transcriptional factor which positively regulates Cdk4 nuclear distribution and therefore cell cycle progression from G1 to S-phase. For this reason we decided to bring a little modification to our Task-2 by pushing forward the idea that RCP may acts as a potent regulation of cell junction components such as E-cadherin. Our results showed that RCP overexpression increase the endogenous pool of E-cadherin at plasma membrane in TM15 cells (Fig. 3). These data argue that increasing of RCP expression led into decrease of E-cadherin internalization and would directly affect the cell junction properties of E-cadherin.

Minor modification of Task-2.2a and Task-2.2b. In the meantime, we have subjected the indicated cell lines included RCP condition to internalization and detection of E-cadherin, a well-known adherent junction and epithelial mesenchymal transition marker. Our result indicates that overexpression of RCP promotes an elevation of the stability of E-cadherin protein at cell surface. These results were obtained by immunofluorescence microscopy technique. Thus, we take the decision to continue our study by investigating the functional role of RCP in
regulating cell junction in ErbB2-driven model.

**2c. Evaluate whether loss of RCP or overexpression of RCP-GFP in primary ErbB2 tumor cells modulates endogenous activated ErbB2 endocytic. Tracking of cell distribution of ErbB2 using endosomal markers.**

Curiously, we detect no significant change in terms of internal distribution of ErbB2 in both RCP-overexpressed and RCP-depleted conditions. However, we assessed immunostaining of autophagosomes marker using immunofluorescence imaging approach in similar RCP cell conditions as in Task-2.2a/2b. Our preliminary results indicated that at endogenous level the ErbB2 protein is co-localized with LC3B-positive vesicles which is a well-defined autophagosome marker. The overexpression of RCP but not loss of RCP promotes an increase of ErbB2 population within these autophagosomes structures (data not shown). Quantification will be performed during Year-2.

**2d. Identification of key molecular component of RCP-ErbB2 pathway. Tumor cell models as in Obj.2 Task-2b will subject to immunoprecipitation of endogenous ErbB2 and LC-MS and peptide sequencing to examine the nature of protein found in complex with ErbB2 in endocytosis program.**

LC-MS peptide sequencing were performed on immunoprecipitated ErbB2 cell lysates and peptide sequencing indicates that endogenous ErbB2 is found to be in complex with Clathrin molecule as well as SNX9 (Sorting Nexin-9). These two candidates were next validates in the following Task-2.2e.

**2e. Validate the function of protein candidates identify as in Obj.2 Task-2d in the modulation of ErbB2 trafficking using RNAi lentiviral approach and similar technique as in Obj. 2 Task-2a. In parallel, monitor the dynamic of ErbB2-Clathrin or –Caveolin-1 complexes by endogenous immunoprecipitation and Western blot analysis.**

In the present Task-2.2e, we were not able to assessed Clathrin-siRNA because knockdown of this protein will destroy the essential endocytotic route that cell lines commonly use to insure down-regulation of transducing signal from cell surface. However, our data demonstrates that overexpression of RCP led into an increase or ErbB2/Clathrin (Fig. 4 left panel) and ErbB2/SNX9 (Fig. 4 right panel) protein complexes suggesting the RCP may drive the endocytotic rate of ErbB2 receptor through Clathrin Coated Pit vesicles.

**2f. Quantification of confocal microscopy and immunoprecipitation experiments. Each co-localization of ErbB2 with different endosomal marker will be translated as the merge of fluorescence signal from Z-stack pictures.**

Quantification of ErbB2 immunoprecipitation will be performed by Image Quant software analysis. This data set will be assessed to an ANOVA statistical analysis. Quantification of our microscopy experiments as well as Western blot analysis are still under processing.

**SOW-Task 3.** Determine the effects of RCP-proficiency and deficiency on Histone-3 methylation in primary ErbB2 tumor cells (Months 2-3): Completion 100%

**3a. Using RCP tumor cell models as in Obj.2 Task-2b assess histone acid extraction and subject intact histones to LC-MS analysis to determine the global profile of histone methylation. A duplicate of each histone sample will assess to tryptic digestion/HPLC/LC-MS and peptide sequencing to detect the degree of methylation on all lysine residues and identification of histone regulators.**

This Task-3a was conducted in collaboration with the Research Institute of immunology and cancerology of Montreal. Histone profiling showed an increase in abundance of methylation on lysine-9 and lysine-27 of Histone-3 (H3meK9) in its amino-terminal tail. To sum, we detect an increase of H3meK9 and H3meK27 in RCP-
overexpressed TM15 cell lines (Fig. 5 left panel).

3b. Validate the method as in Obj. 2 Task-3a by detection of H3meK27 (mono-/di-/and tri-methylation status) and H3meK9 (di- and tri-methylation status) level as well as level of histone regulator candidates by Western blot analysis.

Our results showed a dramatic increase of H3meK9 levels in RCP-overexpressed TM15 cells (Fig. 5 right panel). However, we still try to validate the H3meK27 status in similar condition but as detected a strong background signal by protein analysis we predict that it should be more relevant to use an alternative technique such as immunocytochemistry.

3c. Analysis of mass spectrometry data. Quantification of number of modified peptides which will subclass in different groups of mono-/di- and tri-methylation status and ANOVA statistical analysis will be assessed between different groups.

As presented at figure 5 (left panel), we observed an increase of indicated number of peptides. These peptides are the result of trypsinisation treatment of histone extracts.

SOW-Task 5. Generate and characterize the inducible RCP knockout mouse model (Years 1-3): Completion 50%.

5a. Perform introgression (backcrossing) of the new floxed RCP funder line with FVB genetic background.

During Year-1, we performed embryos implantation having floxed RCP allele within FVB female recipient and then at least four backcrosses. These steps were successfully accomplish and we already started to generate R Cp\textsuperscript{flx/flx}, R Cp\textsuperscript{flx/-} and R Cp\textsuperscript{-/-} and already determined the efficiency of Cre recombinase allele excision by PCR and Western blot. As illustrated at figure 6 our MMTV-Cre/RCP\textsuperscript{+/--} heterozygous mice demonstrated a significant decrease of endogenous RCP expression (Protein immunoblot-Western blot technique).

SOW-Task 6. Characterize the RCP-proficient mouse model (Years 1-2): Completion 75%.

6a. Cross MTB and MTB-NDL mice with Tet-his-RCP-IRES-GFP (RCP) mice.

This Task 6a is well completed. We successfully generated RCP-MTB bigenic mice and RCP-MTB-NDL trigenic mice and already started experimental cohorts. The major caveat when we worked with different breeding pairs of transgene bearing animal is the low efficiency to get high rate of bigenic or trigenic because of Mendelian genetic ratio.
6b. Monitor mammary gland outgrowth of RCP-MTB mice. Induction or not with doxycyclin of RCP-MTB. Mammary glands extraction, H&E and IHC techniques will assess after 4, 6, 8 and 10 weeks after date of birth. 5 mice per group 40 mice in total.

To elucidate the function of RCP in ErbB2-driven tumorigenesis, we established a bi-genic RCP-MTB mouse strain that consists of a tetracycline inducible RCP (pTet/His-RCP/IREs/GFP) crossed into a MMTV-LTR/rtTA mouse (Fig 6A). In this model, RCP expression is activated by the mammary epithelial expression of rtTA following oral administration of doxycycline. To examine the role of RCP in mammary ductal development, we induced a cohort of 3 week old RCP-MTB mice with doxycycline, collected the mammary glands at 8 weeks of age and performed whole mount analysis. The results showed that induction of RCP led to a decrease in the number of terminal end buds (TEBs) (Fig. 6B) concomitant with increased RCP-GFP transgene expression in mammary epithelial cells (Fig. 6B). Thus, RCP perturbs virgin mammary gland development.

**Objective 3*. Training and Course biology (Years 1-2 and 3)**

<table>
<thead>
<tr>
<th>UC Davis Extension Pathobiology of the mouse course tier-1A.</th>
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</table>

The course was not available as a function of our schedule time. I will retry for the Year-2. However, our lab was the host of the prestigious visit of the director of this course Dr Cardiff where I got special meeting and discussion regarding our own murine material in terms of breast cancer tissues.

* In the previous SOW, I put Objective 5, but actually its 3.

### 3.2.2. Opportunities for training and professional development

**Professional development-1.**
Throughout interaction with my mentor, Dr William Muller, I was subjected to manage the present research proposal including some minor modification of the proposal. For example, with the approval of Dr Muller, I changed the focus of the rationale. In the regards of the endocytosis study in ErbB2-driven model, we found more relevant to investigate the status of E-cadherin instead of the tyrosine kinase receptor of interest by itself. Again under Dr Muller supervision, we mutually concluded that our model will provide more relevant impact results to insure future publication in high impact factor journal.

**Professional development-2.**
During the Year-1 I optimized a protocol relative to the specific isolation of histone from cell lysates, mammary glands and breast tumors collected from murine model. I accomplished this protocol optimization in collaboration with Dr Eric Bonneil from IRIC institute, Montreal, Canada.

**Professional development-3.**
During the Year-1 was attended to Terry Fox Research Institute 5th Annual Scientific Meeting, Montreal and the 10th Conference on Signaling in Normal and Cancer Cells. Banff, Alberta, Canada. These conferences improved my knowledge and was useful established connections Dr Anne-Marie Mes-Masson who held director position at the CHUM-Research Center in Montreal, Canada. Dr Mes-Masson will collaborate for human tissue microarray analysis regarding the RCP status in Her2-positive breast cancer patient.

**Professional development-4.** I was assisted by Ms. Julie-Marie Livingstone a research associate form Dr Mike Hallett (Contributing author of our RCP manuscript), a bioinformatic laboratory at McGill University, Montreal, Canada, to learn how to analyses gene expression profile using bioinformatics.

### 3.2.3. Dissemination of the results.
Nothing to Report.
3.2.4. Year-2

Based on previous SOW, I briefly described the Year-2 plan.

SOW-Task-1. During Year-2 I will follow the ACURO animal appendix as fulfilled and officially approved for acceptance to use mouse models. According to guidelines and regulations of Canadian Council on Animal Care I will complete the renewal of my animal certificate by each January 1st of each year, here referred to january 1st, 2015 coordinated with Ms Susanne Smith, Director of animal compliance office, McGill University.

SOW-Task-2 a-f: This task was slightly modified during Year-1 for impact reason. Briefly, I was supposed to investigate the endocytotic status of ErbB2 in RCP-established cell lines. However, our results demonstrated that murine as well as human breast cancer cell lines harbor a high basal enrichment of ErbB2 in clathrin coated vesicles whereas the overexpression of RCP led into a slightly increase of ErbB2 in these endocytotic structures. In parallel, we have monitored the EMT under similar RCP overexpressed and depleted conditions. Our findings showed that overexpression of RCP led into a drastic increase at cell surface of E-cadherin, a well-known EMT marker, is highly stabilized at cell surface. Our conclusion regarding these observations was to redirect our in terms of protein target for endocytosis study. Importantly, one major limit factor in task-2 was to established RCP-proficient and deficient cell lines using TM15, BT474 as well as SKBR3 cell lines background. However, these experimental procedures were fully completed during Year-1.

SOW-Task-3a-c: During the Year-1, we completed the task 3a. However, we have to continue task 3b to identify some histone regulators within mass spectrometry raw data relative to specific modulation of methylation of H3K9 and H3K27. The task 3c is fulfilled.

SOW-Task-4. Have to start IP injection of the anti-ErbB2 antibody into athymic mice subjected to mammary fat pad transplant as well as tail vein injection of murineTM15 and human BT474 and SKBR3 RCP-ErbB2 established cell lines.

SOW-Task-5a-g. Have to continue tumor onset experimental cohort of RCPKO mice having NIC (MMTV-neuNDL-IRES-Cre oncogene background). Until now at end point stage of tumor progression, statistical analyses showed no significant changes between RCP+/+ -NIC, RCP+/−-NIC and RCP−−-NIC mice. However, as demonstrated at figure X, I at early time point conditional deletion of RCP led into drastic mammary tumor transformation. In order to validate this oncogene-like tumor development I will subject mammary tumors to histological Ki67, TUNEL and Cleaved-Caspase-3 staining in order to monitor the cell proliferation status. Additonally, I am collecting wholemount mammary gland along the Year-2 different RCPKO cohorts.
SOW-Task-6a-e: This task includes the characterization of RCP-proficient transgenic mouse model. Along the Year-1, I progressively completed this objective and generate a substantial numbers of data to produce a remarkable manuscript embedding essentially the role of RCP in ErbB2-mediated mammary tumorigenesis and metastasis formation. Overall, the fully completion of the present task-6 positively influence the impact of my post-doctorate training by the eminent acceptance of the aforementioned manuscript.

IMPACT

During the Year-1 of this research proposal, I generate a complete manuscript wrapping all results relative to the role of RCP in ErbB2-mammary tumorigenesis as well as metastasis formation. The manuscript is untitled: Rab Coupling Protein (RCP) suppresses ErbB2 mammary tumor progression through the induction of cellular senescence program. In summary this manuscript demonstrates that RCP acts as tumor suppressor in ErbB2-driven model by negatively regulating both EMT and cell proliferation. Additionally, we consolidated our results and showed that RCP insure the link between the cell junction dynamic and induction of senescence through the modulation of ZONAB, a transcriptional factor which is known to interacts with the tight junction marker ZO-1 and demonstrate abilities to translocate from cell surface into the nucleus to regulate the transcription of proliferation markers such as ccdn1 and pcna.

Furthermore, I inspected three different genomic human studies and highlighted that expression of RCP is inversely correlated with Her2 status where RCP can actually be used as a potent prognostic marker in Her2-positive breast cancer patient.

Impact on other disciplines

Nothing to Report.

Impact on technology transfer.

Nothing to Report.

Impact on society beyond science and technology.

Nothing to Report.

Section: Changes / problems

SOW-Task-2. As it is aforementioned I made a slight and minor modification regarding the accomplishment of the proposed Task-2 in the original Statement of Work. Our preliminary data regarding the role of RCP in the regulation of the endocytotic status of ErbB2 show no relevant result. However, as we monitor the cell junction dynamic in RCP overexpressed and deleted conditions I detected that RCP highly modulate E-cadherin status, which is a gold standard in the understanding of the EMT progression. For this reason I slightly modified the Task-2 by targeting E-cadherin instead of ErbB2. However, only this targets change and not the breast cancer model. Again, I make this changes to maintain the idea to develop a high impact manuscript whereas the role of RCP in endocytotic status of ErbB2 shows no significant changes.

This task was slightly modified during Year-1 for impact reason. Briefly, I was supposed to investigate the endocytotic status of ErbB2 in RCP-established cell lines. However, our results demonstrated that murine as well as human breast cancer cell ines harbor a high basal enrichment of ErbB2 in clathrin coated vesicules whereas the overexpression of RCP led into a slightly increase of ErbB2 in these endocytotic structures. In parallel, we have monitored the EMT under similar RCP overexpressed and depleted conditions. Our findings showed that overexpression of RCP led into a drastic increase at cell surface of E-cadherin, a well-known EMT marker, is highly stabilized at cell surface in Our conclusion regarding these observations was to redirect our in terms of protein target for endocytosis study. Importantly, one major limit factor in task-2 was to established RCP-proficient and deficient cell lines using TM15,
BT474 as well as SKBR3 cell lines background. However, these experimental procedures were fully completed during Year-1.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Products

Nothing to Report

Journal publications

To be Re-submitted in March, 2015

Pierre-Luc Boulay, Julie-Émilie Huot-Marchand, Cynthia Lavoie, Julie M. Livingstone, Laura Jones, Shirley Campbell, Dongmei Zuo, Jim C. Norman, Gordon Mills, Eric Bonneil, Mike Hallet, Morag Park and William J. Muller. Rab Coupling Protein (RCP) suppresses ErbB2 mammary tumor progression through the induction of cellular senescence program.

Books or other non-periodical, one-time publications

Nothing to report.

Other publications, conference papers, and presentations

2014 The 10th Conference on Signaling in Normal and Cancer Cells. Banff, Alberta, Canada.
2014 Terry Fox Research Institute 5th Annual Scientific Meeting, Montréal.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Product-1. During the Year-1 we generated transgenic mice that express GFP tagged RCP under the transcriptional control of a Tetracycline promoter that can be inducibly expressed in the mammary epithelium MMTV/Tet repressor transgene (MTB) and doxycyclin. To evaluate whether inducible expression of RCP can impact on breast cancer such
as the Her2-postive subtype, we interbreed the mice carrying the inducible RCP transgene with transgenic mice expressing a murine ErbB2KI(NDL) strain.

Product-2. In order to complete the _in vivo_ characterization of RCP in ErbB2-driven model, we generated during Year-1 and Year-2, a loss of function RCP model which consist in Cre recombinase system. To end this, we crossed mice bearing a conditional RCP allele with a MMV/ErbB2/IRES/Cre model.

**PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

<table>
<thead>
<tr>
<th>Name:</th>
<th>Julie-Émilie Huot-Marchand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Role:</td>
<td>Research assistant</td>
</tr>
<tr>
<td>Nearest person month worked:</td>
<td>1 month</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Ms. Huot executed experimental procedures regarding</td>
</tr>
<tr>
<td>Name:</td>
<td>Cynthia Lavoie</td>
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<tr>
<td>---------------------</td>
<td>-----------------------------------------------------</td>
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<tr>
<td>Project Role:</td>
<td>Animal Health technician</td>
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<tr>
<td>Nearest person month worked:</td>
<td>2 months</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Ms. Lavoie practiced mammary fat pad injection of RCP/ErbB2 cell lines. Ms. Lavoie managed the floxed -RCP X FVB backcrosses, maintain ErbB2/NIC, ErbB2/neuNDL and MTB mouse strains.</td>
</tr>
<tr>
<td>Funding Support:</td>
<td>Dr Muller CIHR Canadian grant</td>
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<thead>
<tr>
<th>Name:</th>
<th>Dongmei Zuo</th>
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<tbody>
<tr>
<td>Project Role:</td>
<td>Research associate</td>
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<tr>
<td>Nearest person month worked:</td>
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<tr>
<td>Contribution to Project:</td>
<td>Dr Zuo performed 3D cell culture using Gel Trek matrix.</td>
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<tr>
<td>Funding Support:</td>
<td>Dr Muller CIHR Canadian grant</td>
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<tr>
<th>Name:</th>
<th>Julie-Marie Livingstone</th>
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<tbody>
<tr>
<td>Project Role:</td>
<td>Bioinformatic expert</td>
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<tr>
<td>Nearest person month worked:</td>
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</tr>
<tr>
<td>Contribution to Project:</td>
<td>Ms. Livingstone practiced bioinformatic analyses of clinical status of RCP using human genomic data sets.</td>
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<tr>
<td>Funding Support:</td>
<td>Dr Hallet CIHR Canadian grant</td>
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<tr>
<th>Name:</th>
<th>Shirley Campbell</th>
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<tr>
<td>Project Role:</td>
<td>Post-doctorate fellow</td>
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<tr>
<td>Nearest person month worked:</td>
<td>1 month</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Dr. Campbell accomplished cDNA cloning pTet-RCP-IRES-GFP</td>
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<td>Funding Support:</td>
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<tr>
<th>Name:</th>
<th>Dr. Gordon Mills</th>
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<tbody>
<tr>
<td>Project Role:</td>
<td>Professor</td>
</tr>
<tr>
<td>Nearest person month worked:</td>
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<tr>
<td>Contribution to Project:</td>
<td>Because of his expertise in terms of GTPases, Dr Mills, provide some key important input such as investigation of E-cadherin and Claudin-7 in our RCP-MTB-NDL east tumor specimens.</td>
</tr>
<tr>
<td>Funding Support:</td>
<td>MD-Anderson Cancer centre institute</td>
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Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners

Applicable
SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Nothing to report

QUAD CHARTS: Nothing to report.

APPENDICE I: Abstracts

Abstracts: 2014

- Terry Fox Research Institute 5th Annual Scientific Meeting, Montréal*
- The 10th Conference on Signaling in Normal and Cancer Cells. Banff, Alberta, Canada*
Amplification and elevated expression of Rab Coupling Protein (Rab11FIP1/RCP) has been noted in 10-25% of primary breast cancers and was further correlated with high metastatic grade and poor clinical outcome. Nevertheless, the role of RCP in ErbB2-positive breast cancer remains elusive. In this study, we report that RCP plays a critical role in modulating ErbB2 dependent mammary tumor progression and metastasis. To directly evaluate the RCP in ErbB2 mammary tumorigenesis, we have derived transgenic mice that inducibly express RCP in the mammary epithelium and interbreed these mice with separate strain expressing an MMTV activated ErbB2 transgene. Induction of RCP expression in the ErbB2-driven model results in a tumor onset delay in addition to a drastic defect in metastasis formation. Furthermore, elevated RCP expression promotes a significant decrease in cell proliferation that is associated with a Senescence Associated to Heterochromatin Foci (SAHF) cellular senescence phenotype. In addition, using in vitro 3D-cell culture model of ErbB2 positive breast cancer, we showed that RCP is essential to the maintenance of adherens junctions by regulating E-cadherin endocytosis. Conversely depletion of RCP in ErbB2 positive cell lines leads to a significant increase in the number of lung metastases. Consistent with the importance of RCP as negative regulator of ErbB2 positive breast cancer progression, gene expression profile analysis of human breast tumors showed that the expression of RCP is inversely correlated with ErbB2 levels. Taken together, these observations argue that RCP acts as a tumor suppressor in ErbB2-driven breast cancer progression.

Figure 1. Characterization of RCP overexpression and RCP knockdown in ErbB2/neuNT TM15 cell lines.
A. TM15 cells were derived and were lentivirally transduced with either a shRNA-control or shRNA-RCP or retrovirally transduced with either a pMSCV-empty vector (EV) or pMSCV-His-RCP. Clones were pooled and assessed by Western blot analysis for RCP, His-tag and β-Actin levels. B. Quantification of data presented in A. **P<0.01 are values compared to the shRNA-control (shCon) condition. C. RCP/ErbB2/neuNDL cells were equally seeded at 1X10⁴ cell density in a 10 cm dish and left to grow for 7 days. Results are expressed as the number of cells,

Figure 2. RCP controls cell migration in ErbB2/neuNT-mediated cell migration.
TM15 cells were subjected to Boyden chamber assay. TM15 cell lines were serum starved, plated onto the upper chamber and then treated or not with FBS 10% and left for 6 and 16 hours for cell migration. Quantification of these experimental results are the mean ± SEM of six independent experiments. ††p<0.01; *p < 0.05.
**APPENDICE II: Figures and legends**

**Figure 3.** RCP modulates E-cadherin internalization.

TM15 cells were serum starved for 16 hours, incubated with anti-E-cadherin (E-cad) antibody on ice for 30 minutes, fixed and stained for secondary Alexa-Fluor 488 antibody and counterstained with Hoescht. These images are representative of at least 30 cells observed in 2 independent experiments. Scale bar, 10 μm.

**Figure 4.** Overexpression of RCP increased association between ErbB2/Clathrin and ErbB2/SNX9 protein complexes.

Control (EV) and RCP-overexpressed (His-RCP or RCP) TM15 cell lines were lysed and then assessed for endogenous ErbB2 immunoprecipitation. ErbB2 was immunoprecipitated and interacting RCP and Clathrin (left panel) as well as SNX9 were
detected using Western blot analysis. These results are the mean ± SEM of 3 independent experiments.

Figure 5. Overexpression of RCP in ErbB2/enuNT TM15 cells enhances methylation of Histone-3 at lysine-9. TM15 cells were lysed and subjected to histone extraction and mass spectrometry coupled to proteomic analyses (left panel). Results are expressed as the number of peptides related to specific methylation or acetylation status on lysine (K)-9 and -27 of Histone-3 (H3) (left panel). TM15 cells were subjected to histone extraction and endogenous levels of H3meK9 and Histone-3 were detected by Western blot technique using specific antibodies. These results are the mean ± SEM of 3 independent experiments.
Figure 6. Floxed RCP allele model.

The MMTV-Cre/RCP<sup>+/+</sup> and MMTV-Cre/RCP<sup>-/-</sup> mouse strains were subjected to genotyping using DNA isolated from tail DNA extract (Genotyping labeling). We collected mammary gland and monitored the deletion of RCP using Western blot technique. RCP and β-Actin levels were detected using specific anti-RCP and anti-β-Actin antibodies.

Figure 7. RCP-inducible model.

A schematic of the inducible-RCP/MTB mouse model—A. The RCP mouse strain was crossed with the MMTV-rtTA strain (MTB). B. The RCP-MTB mice were either treated or not with doxycycline (dox) from 3 weeks of age. Mammary glands were collected at 8 weeks of age and GFP expression was assessed using fluorescence microscopy. To monitor mammary gland outgrowth, specimens were stained with hematoxylin and imaged with light microscopy (Scale bar represents 100 µm). Number of terminal end buds (TEBs) was counted manually and data were expressed as the number of TEBs. Significance was determined by student t-test **P<0.01 are values compared to the control (RCP-MTB without dox). The number of animals per experimental condition are; 8 weeks old RCP-MTB without dox n=6, 8 weeks old RCP-MTB with dox n=6.
**APPENDICE: Experimental procedures**

*Antibodies and reagents*—The Clathrin (Heavy Chain) antibody was from Santa Cruz Biotechnology. The E-cadherin antibody was purchased from Calbiochem. Rab11FIP1/RCP and β-Actin antibodies were purchased from Sigma Aldrich. The ErbB2 antibody was purchased from DAKO. The His-tag antibody was purchased from Oncogene Research. All reagents used for IF and Western blot were purchased from Biobasic, Bioshop and Sigma Aldrich.

*Transgenic mice*—The inducible RCP construct was engineered by cloning the His-RCP fragment (pcDNA3.1 cut with HindIII+Xba1) into a pTet-IRESGFP plasmid using EcoRV restriction enzyme sites. The pTet-His-RCP-IRESGFP DNA plasmid was linearized using the PvuI enzyme and transgene integration was performed following standard techniques using the embryonic stem cells line R1. Embryonic stem cell clones were selected to generate chimeras by the McGill Transgenic Core Facility. The mice were introgressed in a FVB/N background over six consecutive generations. Females were bred with male MTB (MMTV-rtTA) mice and treated with doxycycline to induce RCP expression within the mammary epithelium. All mice were housed in the animal facility of the Rosalind and Morris Goodman Cancer Center and all experiments were performed according to the animal care guidelines at the Animal Resource Centre of McGill. Once a week, mammary tumors were detected via physical palpation and measurements were quantified using caliper measurement. Animals were sacrificed 8-weeks following initial palpation.

*DNA Plasmids and shRNAs*— pcDNA3.0-His-RCP (a gift from Jim Norman) was cut with HindIII+Xba1 restriction enzymes and then the His-RCP insert was subcloned into pMSCV-hypgro (Clonetech) using Hpa1 sites to generate pMSCV-hygro-His-RCP. Small hairpin RNAs (shRNAs) control (shRNA-GFP) or against RCP (shRNA-RCP) were obtained from Open Biosystems.

*Cell culture*—ErbB2/neuNT(TM15-clone 6 and 10)-derived tumor cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FBS and SingleQuots (Clonetics). Human breast cancer cell lines SkBr3 and BT474 (American Type Culture Collection) were maintained according to recommendations. ErbB2/neuNT (TM15 clone-6), SkBr3 and BT474 cell lines expressing shRNA-RCP or shRNA control (Open Biosystems) were generated using 293T cells and lentivirally transduced according to the manufacturer's instructions followed by puromycin selection (4 μg/mL; Sigma-Aldrich). A similar subset of cell lines overexpressing pMSCV-hygro-His-RCP or pMSCV-control (empty vector) were generated using 293VSV cells retrovirally transduced according to the manufacturer’s recommendations followed by hygromycin selection (500 μg/mL; Wisent).

*Cell proliferation assay*—RCP/ErbB2 infected cells were plated at a cell density of 1 X 10^4 cells in a 10 cm dish and left to grow for 7 days. At each indicated time point, cells were trypsinized and counted and assessed for ANOVA statistical test.

*Cell migration assay*—TM15 cell lines were serum-starved overnight and seeded into Boyden chambers (24-well inserts with 8-μm pore collagen-coated membranes). One hour after plating, cells were stimulated or not with FBS 10%. After indicated times, cells were fixed using paraformaldehyde (4%) for 20 min and incubated with crystal violet (0.1% in 20% MeOH; overnight). Membranes were washed five times in dH2O, and cells were removed from the upper chamber, leaving those that migrated through the membrane to the lower chamber. Cells were counted and assessed to ANOVA statistical analysis.

*Internalization assay*— TM15 cell lines were subjected to E-cadherin internalization assay. For labeling of the E-cadherin, cells were washed once and incubated with E-cadherin antibody which recognized extracellular epitope of E-cadherin for 30 min on ice. For internalization experiments, cells were simply incubated at 37 Celsius for indicated times and fixed. Cells were incubated with a secondary antibody coupled to a 488 Alexa-Fluor. Cells were counterstained with DAPI. Confocal images were acquired using a Zeiss LSM-510 META laser scanning microscope (Carl Zeiss, Oberkochen, Germany).
**Western Blotting**— Frozen tissues and cells were processed in TGH buffer supplemented with protease inhibitors and 1 nm of sodium orthovanadate. Tumor and cell lysates were solubilised at 4 °C for 30 min. Some subsets of tumors were subjected to cell fractionation as described [18]. Briefly, frozen tumors were solubilised in 100 μl of Buffer I (160 mM sodium chloride, 38 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH: 7.4, 1 mM dichloride magnesium and 1 mM ethylene glycol tetraacetic acid) containing protease inhibitors, incubated on ice for 5 min and then followed by centrifugation at 500 g for 10 min, at 4 °C. Nuclear fraction (NF) were obtained by resuspended pellets in buffer II (100 mM 1,4-piperazinediethane sulfonic acid pH:6.8, 300 mM sodium chloride, 300 mM sucrose, 3 mM dichloride magnesium, 1 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol and 0.5% Triton X-100) containing protease inhibitors. Quantification of protein levels was performed using the Bradford protein assay. Eluted proteins were run on polyacrylamide gels and transferred onto polyvinilidene difluoride (PVDF) membranes. Protein expression level was detected using specific primary antibodies, secondary HRP-conjugated antibodies and chemiluminescence was detected using a developer. Quantification of protein levels was performed using ImageQuant 5.2 software.

**Immunofluorescence**— TM15 cells stably expressing the Empty vector (EV) or His-RCP and depleted in RCP protein (shRCP) were fixed using paraformadehyde (4%), and E-cadherin and Clathrin were successively detected using specific antibodies coupled to Alexa-fluorophores solubilized in permeabilizing media (MEM; 0.1% BSA, 10 mM HEPES, 0.05% saponin). Cells were counterstained with DAPI. Confocal images were acquired using a Zeiss LSM-510 META laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

**Coimmunoprecipitation Experiments**— ErbB2/neuNT TM15 cells were solubilized in 300 μl of TGH buffer (pH 7.3, 1% Triton, 10% glycerol, 50 mM NaCl, 50 mM HEPES, 5 mM EDTA) containing protease inhibitors (4°C for 1 h). Lysates were centrifuged at 10,000 rpm for 5 min, and equal concentrations of soluble protein were incubated with the ErbB2 antibody and protein G-PLUS agarose beads. The beads were washed, and bound proteins were eluted into 20 μl of SDS sample buffer containing 5% β-mercaptoethanol and heated to 95°C for 5 min. Interacting protein were detected by immunoblot analysis using specific antibodies.

**Histone extraction and mass spectrometry**—Histones were extracted from cell lysates using acid extraction protocol as described [22], except that the lysate were homogenise in PBS solution by passing the sample three times through a 25 gauge needle. Extracted histones were run on a polyacrilamide gel and histones bands were cut and shrinked in 50% ACN and reconstituted in 50 mM ammonium bicarbonate with 10 mM TCEP and vortexed for 1 h at 37°C. Chloroacetamide was added for alkylation to a final concentration of 55 mM. Samples were vortexed for another hour at 37°C. 1 μg of trypsin was added to the solution and the digestion was performed for 8 h at 37°C. Peptide extraction was conducted with 90% ACN. Extracted peptide samples were dried out and solubilised in ACN 5% formic acid (FA) 0.2%. Samples were loaded on a homemade C18 precolumn (0.3 mm i.d. x 5 mm) connected directly to the switching valve and separated on a homemade reversed-phase column (150 μm i.d. x 150 mm) with a 56-min gradient from 10–60% acetonitrile (0.2% FA) and a 600 nl/min flow rate on a NanoLC-2D system (Eksigent) connected to an LTQ-Orbitrap Elite (Thermo Fisher Scientific). Each full MS spectrum acquired with a 60,000 resolution was followed by 12 MS/MS spectra, where the 12 most abundant multiple charged ions were selected for MS/MS sequencing. Tandem MS experiments were performed using collision-induced dissociation in the linear ion trap. The data were processed using the Mascot 2.4 (Matrix Science) and the IPI Mouse database v. 3.54(111974 sequences). Tolerances on precursors and fragments were 15 ppm and 0.5 D, respectively. Variable selected post-translational modifications were carbamidomethyl (C), oxidation (M), deamidation (NQ), methyl (KR), dimethyl (KR), trimethyl (KR) and acetyl (KR).

**Statistical Analysis**— Statistical analysis was performed using either a one-way or two-way ANOVA followed by a Bonferroni multiple comparison test as well as Student-T test using GraphPad Prism (version 4.0a, San Diego, CA).