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### 14. ABSTRACT
The objective of this project is to develop prognostic markers for prostate cancer (PCa) and identify potential therapeutic targets. Specific aims are: 1) To profile bone metastasis samples to identify genomic alterations of PCa metastases that can be retrieved in their corresponding primary tumors, 2) To evaluate the prognostic value of specific genomic alterations in localized primary tumors with clinical follow-up database, 3) To ascertain the relevance to disease progression of genes residing in genomic alterations of prostate cancer metastases. PCa bone metastases resection material collection is currently ongoing (AIM1). Chromosome 10q23 (PTEN) deletion was assessed on a TMA of a subset of primary PCa samples (hemizygous and homozygous deletion in 41 and 11% respectively), and its prognostic value is being confirmed on the full McGill TMA. Specificity of probes for 8q24.21 and 8q24.3 (MYC and PTK2) was confirmed and will be tested on a subset of primary PCa samples (AIM2). Under AIM3, downregulation of GABARAPL2, a gene located in a chromosomal region deleted in PCa metastases, showed increase in autophagy in a PCa cell line and reduced growth. Another target, which gene is located at 16p13 showed to be gained in PCa metastases, is also being assessed. So far, our results suggest that genomic alterations may serve as prognostic markers that would improve the clinical management of PCa.

### 15. SUBJECT TERMS
prostate cancer, genomic alteration, chromosome gain and deletion, fluorescence in situ hybridization (FISH), prognostic markers, biomarkers, tissue microarrays, autophagy

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1. INTRODUCTION:

Prostate cancer (PCa) is a very heterogeneous disease ranging from indolent to metastatic deadly disease. A major challenge in clinical management of PCa is to predict, at the time of diagnostic, the outcome of the tumor and consequently choose the best treatment option for each patient. Current markers - preoperative serum prostate specific antigen (PSA) levels, tumor stage and biopsy Gleason score (GS) - cannot accurately predict individual patient outcome. For advanced and metastatic disease there is no curative treatment. Genomic profiling studies have identified specific genomic alterations such as chromosome gains and deletions associated with aggressive PCa [1] [2] [3]. This research project aims to develop new prognostic markers and identify the relevant tumor genomic alterations associated with disease progression.

2. KEYWORDS:

Prostate cancer, castrate resistant prostate cancer (CRPC), DNA copy number alteration, chromosome gains and deletions, fluorescence in situ hybridization (FISH), biomarkers, tissue microarray (TMA), colony formation assay, cell growth, autophagy.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The hypothesis of the project is that specific genomic alterations, determinant of tumor behaviour, could be detected in primary tumor and predict clinical outcome. The objective is to develop prognostic markers for PCa and identify potential therapeutic targets. To that goal we proposed the following specific aims:

- Specific AIM 1: To profile bone metastasis samples to identify genomic alterations of PCa metastases that can be retrieved in their corresponding primary tumors;
- Specific AIM 2: To survey selected cohorts of clinical prostate samples for genomic changes and evaluate their prognostic value;
- Specific AIM 3: To ascertain the relevance to disease progression of genes residing in genomic alterations of PCa metastases.

Our progress is summarized below, with figures supporting the data shown in the appendices (section 9). Please note that all data presented in this report are unpublished.

What was accomplished under these goals?

Specific AIM 1
- Collection of PCa bone metastasis sample from patient in McGill cohort.
In order to increase access to PCa bone metastasis samples (Task 2 in Statement Of Work (SOW)), collaboration with McGill orthopaedic surgeon Dr. Michael Weber was recently established. One case of PCa bone metastasis was collected after resection of the metastatic tumor in the vertebrae pedicle and lamina, as well as rib head. From this first sample, different portions of the resected tumor were i) fresh frozen, cut and H&E stained for visual assessment of tumor infiltration (Figure 1), while other portions are ii) kept frozen at -80°C for future extraction and profiling of DNA and RNA, or iii) are being decalcified in order to slice the tissue to perform fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC). Arrangements with Dr. Weber include introduction of a detailed procedure for collection of samples in operation room, as well as the use of a pager to let us immediately know when a patient is scheduled for bone metastasis resection. With this newly set up procedure and collaboration, we are confident to gain access and collect bone metastasis material as they are processed at the McGill University Health Centre at a much faster rate.

Specific AIM 2
In our previous Progress Report, we showed that 16p13.3 genomic gain in primary PCa samples was significantly associated with clinico-pathological features of aggressive PCa. According to Task 3 of the SOW, we initiated the assessment of prognostic value of other genomic alterations (8q24, 10q23) found in metastases and retrieved in primary tumors, which may serve as predictors of progression.

-FISH analysis of 10q23 deletion on primary radical prostatectomy samples on the McGill Urology TMA with complete follow-up.

We first assessed the specificity of the 10q23 FISH probe (Figure 2 A-E) on normal lymphocyte metaphases and on different PCa samples harboring a hemizygous, homozygous or no deletion. Then, this specific PTEN probe was used on the McGill Urology TMA of 400 cases, and FISH analysis of the 46 cases probed so far (Figure 2 F) detected hemizygous PTEN deletion in 19/46 (41%) and homozygous PTEN deletion in 5/46 (11%) of the primary radical prostatectomy samples. Hybridization and analysis of this 10q23 probe on the entire cohort is currently being performed.

-FISH probe validation of 8q24 (MYC, PTK2).

Another previously identified genomic alteration in PCa metastases is gain of chromosome 8q24 [2], with known oncogenes MYC (8q24.21) and PTK2 encoding FAK (8q24.3), which were other targets listed in Task 2 of specific AIM 2. Specific probes were generated from BAC clones and hybridized on normal lymphocyte metaphases, with their commercially available centromere control. Figure 3 A) shows representative images of both probes with their centromere control on normal lymphocyte metaphases, while B) shows same probes hybridized on 2 separate tissue slides of the same primary PCa sample. These probes will be hybridized on the
McGill TMA of 400 cases of prostatectomy with clinical follow-up to determine their prognostic value.

Specific AIM 3
-Effect on cell growth of GABARAPL2 modulation in LNCaP, 22Rv1 and PC-3.

In our previous Progress Report, under AIM 3 Task 1 of the SOW, we showed that modulation of GABARAPL2 by lack vs. gain (siRNA and overexpression, respectively) did not have an effect on autophagosome formation in 22Rv1 and LNCaP cells, as suggested by LC3-II/LC3-I ratio in Western blotting (marker of autophagy), but that in PC-3 cells, downregulation of GABARAPL2 increased both basal and 24h starvation-induced LC3-II/LC3-I ratios. Cell growth assays showed no effect in 22Rv1 and LNCaP cells, but hadn’t been yet performed on PC-3 cells.

Hence, we wanted to know if this modulation could have an impact on cell growth of PC-3 cells. MTT assay (under basic culture conditions, no starvation) revealed that downregulation of GABARAPL2 with 2 different siRNAs significantly reduced cell growth, and that this effect was rescued by re-expressing GABARAPL2 (Figure 4). This experiment showed for the first time that downregulation of GABARAPL2 in PC-3, but not in LNCaP nor 22Rv1 cells, reduced cell growth.

-Modulation of autophagy with analog of rapamycin (Rad001, also known as Everolimus) in PC-3.

With the interesting results from the previous experiments in PC-3 cells, we wanted to further assess the autophagy modulation by GABARAPL2 with a known inducer, the rapamycin analog Rad001 [4]. To determine the optimal concentration to be used, we first incubated PC-3 cells with an increasing concentration of Rad001, from 10 nM to 10 μM, with appropriate vehicle controls (DMSO), and assessed phosphorylation level of p70S6K, a direct target downstream of mTOR that was used as a positive control, as well as autophagy induction by LC3-II/LC/-I ratio. 2.1 μM of Rad001 gave the highest ratio in LC3-II/LC/-I, showing strong induction of autophagy (Figure 5), as reported in the literature [5]. Treatment with Rad001 at all concentrations induced reduction in signal of phospho-p70S6K, as expected.

We then downregulated and overexpressed GABARAPL2 in PC-3 cells, in the context of starvation, Rad001 treatment, or both. MTT assay revealed (Figure 6) that starvation itself induced significant reduction in growth 5 days post-transfection, in all transfection conditions (***= p<0.01). Downregulation of GABARAPL2 by siRNA further decreased cell growth (*= p<0.03) under all treatments. Overexpression of GABARAPL2 rescued that effect, but did not overcompensate. Western blotting for all conditions was performed for GABARAPL2 to confirm downregulation or overexpression, for phospho-p70S6K to confirm inhibitory Rad001 effect on mTOR, and for LC3 isoforms to assess impact on autophagy (Figure 7).
With this experiment, we lack consistency in the LC3-II/LC3-I ratio as we had previously witnessed with Rad001 treatment. Indeed, bottom panel of Figure 5 showed that treatment with Rad001 increased LC3-II/LC3-I ratio, while bottom panel of Figure 7 shows no increase for the same Rad001 treatment, in the control transfection condition (pcDNA-6 + siControl). Articles in the literature have suggested the use of LC3-II antibodies in fluorescence microscopy to assess autophagosome formation, as an alternative to LC3-II/LC3-I ratios in Western blotting [5].

-Generation of stable clones overexpressing ECI1 and colony formation assay.

In parallel to our work on GABARAPL2, we recently started studying ECI1 (enoyl-CoA delta isomerase 1, previously reported as DCI), another relevant gene residing in the minimal region of gain of chromosome 16p13 [6]. Our previous gene expression analysis of various PCa subtypes revealed that along PDPK1, ECI1 was significantly overexpressed in the most aggressive PCa subtype-3, as compared to clinically favourable subtype-1 and normal prostate tissues (Figure 8 A). ECI1, which has not been studied in PCa, is a mitochondrial enzyme involved in beta-oxidation of unsaturated fatty acids, a metabolic pathway involved in cancer cell survival and growth [7]. ECI1 overexpression may thus provide PCa cells a survival advantage. Under AIM3, Task 1, we undertook the assessment of in vitro modulation of ECI1 and its relevance to disease progression.

We started by assessing the endogenous expression of ECI1 in a panel of PCa cell lines (Figure 8 B). Based on this, we chose 2 cell lines with differential basal expression of the ECI1 protein to perform overexpression; PC-3 (low) and 22Rv1 (high). 72 hours post transfection of PC-3 cells with either a pcDNA6-Mock, untagged or V5-tagged ECI1, proteins were harvested and used to assess successful ECI1 overexpression by Western blot (Figure 9 A). In parallel, those transfected PC-3 cells were maintained under blasticidin selection and after 25 days, colonies were visually counted for each transfection conditions. We observed a significantly higher number of colonies in the ECI1 overexpression conditions (Figure 9 B). Results for the same procedure applied to 22Rv1 cells are shown in Figure 9 C) (72 hours post-transfection) and D) (37 days of blasticidin selection).

Figure 10 A) shows Western blotting of 22Rv1 cell lysates from an independent set of Mock, ECI1-untagged or V5-tagged clones assessing stable ECI1 overexpression (with ECI1 or V5 antibodies) after 35 days of selection with blasticidin. Since only 2 ECI-V5 clones were produced in this transfection set, Figure 10 B) shows another set (independent transfection) of ECI1-V5 clones in 22Rv1 cells after 35 days of blasticidin selection.

Generation of stable clones overexpressing ECI1 was done in the same fashion in PC-3 cells (Figure 10 C), whereas only one set of transfection and clone selection was necessary to generate suitable amount of ECI1-untagged and V5-tagged clones. Selected stable clones were then used to perform colony formation assay (Figure 11
A) wherein the ECI1-overexpressing clones gave significantly higher number of colonies as compared to the Mock (p<0.01). Results from 2 separate colony formation assays in Figure 11 B) shows more colonies with untagged or V5-tagged ECI1-overexpressing clones compared to Mock. Western blotting with PC-3 cell lysates from Mock, untagged of V5-tagged stable clones depicting maintained overexpression is shown in Figure 11 C). Those results suggest that ECI1 provides a survival advantage, or at least a faster growth rate.

What opportunities for training and professional development has the project provided?

This project is the work of a PhD (AIM 2-3) and a MSc candidates (AIM 3), with the help of my research assistant (AIM 1-2-3), all of them in my laboratory under my direct supervision (Yogesh Bramhecha, Nishath Syed and Karl-Philippe Guérard, respectively). They all are developing new skills by performing the required experiments to complete the specific tasks related to this project. During their graduate studies, both students are actively learning how to meticulously plan and perform experiments, in order to generate scientifically sound results, a notion that will serve them throughout their career. Also, writing and publishing their results in scientific journals will make them develop interpretation capabilities, as well as make them participate in the dissemination of knowledge.

How were the results disseminated to communities of interest?

The results and analyses that stemmed from the experiments described above are unpublished, but are being written in the form of manuscripts soon to be submitted to peer-review, public-access scientific journals. Sections 6 of this report will show the list of presentations and posters presented in regional and national conferences, by both the graduate students who worked on the project.

What do you plan to do during the next reporting period to accomplish the goals?

AIM 1: To pursue the goal of identifying new genomic signatures in bone metastases and in their corresponding primary tumors (Milestone), we will continue collecting bone metastases resection material to perform gene expression analysis (Task 2) and FISH on both the metastases and their corresponding primary tumors (Task 3).

AIM 2: We will start FISH analyses of 8q24.21 (MYC) and 8q24.3 (PTK2) probes and complete the analysis of 10q23 (PTEN) probe by surveying the complete McGill
Urology TMA of 400 cases of prostatectomy with clinical follow-up (Task 3), to determine their prognostic value (Milestone).

AIM 3: In order to demonstrate the role of target genes in the formation of metastases (Milestone), we will use LC3-II antibodies in fluorescence microscopy to assess autophagosome formation, as an alternative to LC3-II/LC3-I ratios by Western blotting after GABARAPL2 downregulation or overexpression (Task 1). Also, after confirming that GABARAPL2-downregulation stable clones affect cell growth in vitro (Task 2), in vivo metastasis experiments will be performed using BALB/c mice, as described in original proposal (Task 4).

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.
Actual or anticipated problems or delays and actions or plans to resolve them

The Research Institute of the McGill University Health Centre, scattered throughout different centres and hospitals in Montreal, physically moved to a new site in February 2015. This was explained in the "Request for an extension without funds (EWOF)" from October 2015. As we are now established in a new, state of the art building with the most modern research infrastructure, no further delays are expected.

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.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

Publications, conference papers, and presentations

Journal publications.

Books or other non-periodical, one-time publications.
Other publications, conference papers, and presentations.


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

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?

<table>
<thead>
<tr>
<th>Name:</th>
<th>Jacques Lapointe (no change)</th>
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<tbody>
<tr>
<td>Name:</td>
<td>Karl-Philippe Guérard (no change)</td>
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<tr>
<td>Name:</td>
<td>Yogesh Bramhecha</td>
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<tr>
<td>Project Role:</td>
<td>Graduate Student</td>
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<tr>
<td>Researcher Identifier (e.g. ORCID ID):</td>
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<tr>
<td>Nearest person month worked:</td>
<td>12</td>
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<tr>
<td>Contribution to Project:</td>
<td>PhD candidate Yogesh Bramhecha performed experiments related to AIM 2 (FISH) and part of AIM 3 (in vitro functional assays).</td>
</tr>
</tbody>
</table>
Name: Nishath Syed

Project Role: Graduate Student

Researcher Identifier (e.g. ORCID ID): -

Nearest person month worked: 9

Contribution to Project: MSc candidate Nishath Syed worked on GABARAPL2 expression modulation experiments in AIM 3.

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?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

9. APPENDICES:

REFERENCES


Figure 1. H&E staining of PCa bone metastasis sample. Resection of PCa bone metastasis from vertebrae pedicle was performed at McGill University Health Centre. A fresh-frozen cryostat section (10 μm) was stained with Hematoxylin and Eosin (H&E). Black arrows show infiltration of cancer cells within bone structure.
Metaphase FISH with *PTEN* probe

Tumor with No *PTEN* deletion

Tumor with hemizygous *PTEN* deletion

Tumor with homozygous *PTEN* deletion

Tumor with hemizygous & homozygous deletion

**Figure 2: FISH for 10q23 (*PTEN*) deletion.** A) Validation of *PTEN* FISH probe specificity on normal lymphocyte metaphase, showing 1 green and 1 orange signals (yellow arrow head). White arrows show B) normal interphase nuclei with 2 green and 2 orange signals in a PCa tumor with no *PTEN* deletion, C) 2 green and 1 orange signals in a tumor harboring hemizygous *PTEN* deletion and D) 2 green and 0 orange signals in a homozygous *PTEN* deleted case. E) Tumor sample with nuclei harboring hemizygous (white arrows) or homozygous (yellow arrow) deletions respectively. F) FISH analysis detected hemizygous *PTEN* deletion in 19/46 (41%) and homozygous *PTEN* deletion in 5/46 (11%) of the primary radical prostatectomy samples on the McGill Urology tissue microarray analysed so far (n = 46).
Figure 3: FISH probes for 8q24.21 (MYC) and 8q24.3 (PTK2). A) Specificity of MYC and PTK2 FISH probes on normal lymphocyte metaphase. White arrow points to chromosome 8 with specific probes hybridized. B) Same MYC and PTK2 probes hybridized on separate slides of the same PCa patient, showing nuclei from tumor area with 2 green and 2 orange signals.
Figure 4: Effect on PC-3 cell growth of modulation of GABARAPL2. 0, 3 or 5 days after transfection of PC-3 cells with a combination of Mock or GABARAPL2 overexpressing vector and Control or GABARAPL2 siRNAs, cell growth was monitored by MTT assay. 2 different siRNAs against GABARAPL2 (red box and purple cross) showed significant reduction in growth, that was rescued by re-expression of siRNA-resistant GABRAPL2 (orange circle and pink dot). * denotes significant difference in cell viability at T=5 days post transfection with p-value < 0.04 between transfection conditions.
Figure 5: Effect on LC3II/LC3I ratio of increasing concentrations of Rapamycin analog (Rad001). PC-3 cells were treated with Rad001 with concentration ranging from 10 nM to 10 μM. Matched volumes of DMSO served as a control vehicle. 25 μg of protein from the treated cells were assessed for GABARAPL2, LC3-II/LC3-I, S6K total and p-S6K expression by Western blotting probed with their respective antibodies. Relative expression is indicated for all tested proteins (to Control-Vehicles and normalized to actin).
Figure 6: Effect of GABARAPL2 modulation on growth in PC-3 cells with or without starvation, Rad001 or both. 24h post-transfection, cells were treated with either DMSO alone (control vehicle), starvation (phenol red free RPMI media), 2.1μM of Rad001 or combination of starvation and Rad001. MTT assay was done on these cells at three time points, 0, 3 and 5 days post-transfection (or 0, 2 and 4 days post-treatment). * denotes significant difference in cell viability at T=5 days post transfection (or 4 days post treatment) with p-value < 0.03 between transfection conditions. ** denotes significant difference in cell viability at T=5 days post transfection (or 4 days post treatment) p-value < 0.01 between treatment condition (e.g. control vs. starvation).
Figure 7: Effect of GABARAPL2 modulation on autophagy marker in PC-3 cells with or without starvation, Rad001 or both. PC-3 cells were transfected to modulate GABARAPL2 expression. The transfected cells were treated with either DMSO alone (control vehicle), starvation (phenol red free RPMI media), 2.1 μM of Rad001 and combination of starvation and Rad001 for 2 days (48 hours). 25 μg of protein from the treated cells were assessed for GABARAPL2, LC3-II/LC3-I, S6K total and p-S6K expression with Western blot analysis probed with their respective antibodies. Relative expression is indicated for all of the proteins tested (to pcDNA-6 +siControl and normalized to actin).
Figure 8: ECI1 expression in PCa samples and cell lines. A) Cluster analysis of variably-expressed genes in 16p13.3 gain region, modified from Lapointe et al., 2004, where hierarchical clustering distinguishes normal prostate (pink branches) from tumors, the latter further stratified into subtypes-1, -2 and -3 (yellow, blue, purple) based on gene-expression patterns. Expression heatmap demonstrates that ECI1 expression is significantly higher in clinically-aggressive subtype-3 tumors as compared to favorable subtype-1 and normal prostate samples. B) Cells were grown in RPMI-1640 medium supplemented with 10% FBS and assessed for basal levels of expression of ECI1 by Western blot analysis (Novus Biologicals #NBP1-91822). Actin was used as control.
Figure 9: Effects of ECI1 overexpression on PCa cell growth. Western blot analysis confirming overexpression of ECI1 in A) PC-3 and C) 22Rv1 cell lines as assessed by anti-ECI1 and anti-V5 (Life Technologies Cat. no. R96025) antibodies, 72 hours post-transfection. Actin was used as control. Bar graphs represent significant increase in the number of colonies B) per field of view for PC-3 cells after 25 days (5 fields of view per condition, p<0.01, t-test; n=1) and D) per plate for 22Rv1 cells after 37 days of blasticidin selection (percentages relative to Mock, p<0.05, t-test; n=2) for stable ECI1 overexpression (untagged and V5-tagged) as compared to cells transfected with Mock vector.
Figure 10: Generation of stable clones overexpressing ECI1 (untagged and V5-tagged) in PCa cell lines. Western blot analysis characterizing A) different stable 22Rv1 clones overexpressing ECI1. Clones ECI1-2 and -4 have higher ECI1 expression (left panel) as compared to Mock clones. Similarly, V5-tagged ECI1 clones (right panel) V5-1 and V5-3 express higher amount of ECI1-V5 (upper band). V5-2 clone showed no cell growth and was discarded. Antibodies used were anti-ECI1 and Actin as control. Western blot analysis characterizing independent set of V5-tagged ECI1 clones in B) 22Rv1 or C) PC-3 cells using anti-ECI1 (top panels) and anti-V5 (middle panels) antibodies respectively. Percentages show quantification of B) upper ECI1-V5 or C) lower ECI1 band, after normalization to Actin (bottom panels). In B), clone selection plates for Mock and ECI1-untagged were lost to contamination and hence only ECI1-V5 clones (identified with stars) could be selected and evaluated against the Mock-1 and Mock-2 from the previous 22Rv1 experiment (Figure 10A).
Figure 11: Effect on growth of ECI1-overexpressing PC-3 clones. A) Crystal violet staining depicts significant increase in the total number of colonies/well for independant stable PC-3 clones overexpressing ECI1 (untagged i.e. ECI1-1, -2, -3 and V5-tagged i.e. V5-1, -3), as compared to stable Mock clones (Mock-2, -3). B) Bar graph representing significant increase in the mean number of colonies/well for stable PC-3 clones overexpressing untagged and V5-tagged ECI1 respectively (p<0.01, t-test; n=2). * denotes significant difference from Mock-2 and ** from Mock-3 clone. C) Western blot analysis confirming stable overexpression of ECI1 in PC-3 clones as assessed by anti-ECI1 (top panel, Novus Biologicals #NBP1-91822) and by anti-V5 (middle panel, Invitrogen Cat. no. R96025) antibodies respectively. Percentages show quantification of top panel ECI1 band, normalized to Actin (bottom panel), compared to Mock-3.