

AWARD NUMBER: W81XWH-14-1-0266

TITLE: Targeting Signaling to YAP for the Therapy of NF2

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REPORT DATE: December 2016

TYPE OF REPORT: FINAL

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

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OMB No. 0704-0188

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1. REPORT DATE December 2016		2. REPORT TYPE FINAL Report		3. DATES COVERED 30 Sep 2014 - 29 Sep 2016	
4. TITLE AND SUBTITLE Targeting Signaling to YAP for the Therapy of NF2				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-14-1-0266	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Filippo G. Giancotti, M.D., Ph.D. E-Mail: fgiancotti@mdanderson.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Sloan-Kettering Institute for Cancer Research 1275 York Avenue New York, NY 10065-6007				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The goals of this project are to utilize our new knowledge of the mechanism by which loss of Merlin induces tumorigenesis to identify small molecule compounds that block YAP/TEAD-dependent transcription by acting at any step of our newly identified pathway, and to test the preclinical efficacy of lead compounds in xenograft models of NF2. During this grant, we have generated <i>NF2</i> mutant cell lines expressing two different types of YAP reporters and tested their suitability for high throughput screening.					
15. SUBJECT TERMS Neurofibromatosis type 2, Merlin, Hippo-YAP, mTOR, E3 ubiquitin ligase, high through put screening, small molecules, siRNA					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 10	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

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1. INTRODUCTION: The tumor suppressor NF2/Merlin is a multifunctional protein, which shuttles between the cell cortex and the nucleus in a manner reminiscent of the cell adhesion and signaling component α -catenin (1). Following up on a functional cDNA screen that yielded PAK as a mediator of exit from contact inhibition, we found that its target Merlin integrates antithetic signals from cadherins and integrins to regulate cell proliferation (Fig.1) (2).

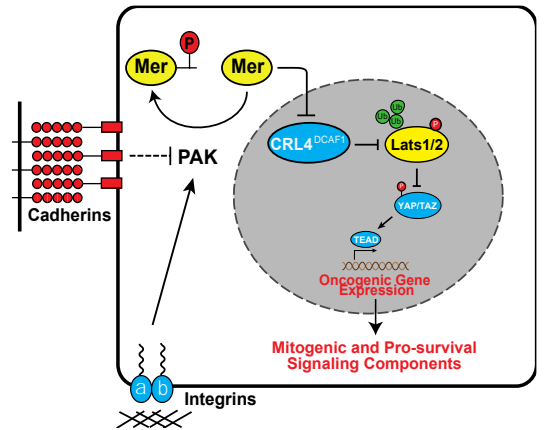


Fig. 1. Merlin-mediated tumor suppression. Contact inhibition or loss of matrix adhesion, inactivate PAK, leading to nuclear accumulation of de-phosphorylated, active form of Merlin, which binds to CRL4^{DCAF1}, blocking its ability to ubiquitinate Lats and thereby drive YAP/TEAD-dependent transcription of mitogenic and pro-survival genes..

Intriguingly, we observed that CRL4^{DCAF1} controls an oncogenic program of gene expression that includes TEAD target genes, suggesting that Merlin controls Hippo signaling by inhibiting CRL4^{DCAF1}. More recently, we found that de-repressed CRL4^{DCAF1} targets Lats1 and 2 for ubiquitylation and inhibition in the nucleus, thus activating YAP-driven transcription and oncogenesis. Genetic epistasis experiments provided evidence that this oncogenic pathway sustains the oncogenicity of NF2 mutant cells and analysis of clinical samples confirmed its activation in human NF2 mutant tumors [Li et al., Cancer Cell 2014] (4). In parallel, it has become clear that Merlin can suppress mitogenic signaling also by binding to Angiomotin (AMOT, AMOTL1, or AMOTL2) at the cell cortex (5). In order to identify targets for therapeutic intervention in NF2, we are continuing to define the complex mechanisms through which loss of Merlin activates mitogenic signaling by using somatic cell genetics and biochemistry. In addition, we have initiated high-throughput screens with siRNA and small molecule libraries to identify targets and drugs for therapeutic intervention.

2. KEYWORDS: Neurofibromatosis type 2, Merlin, Hippo-YAP, mTOR, E3 ubiquitin ligase, high through put screening, small molecules, siRNA.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The overall goal of this grant is to conduct high-throughput cell-based screens to identify lead compounds that inhibit YAP/TEAD-dependent transcription.

Task 1. The FC1801 will be stably transduced with a Firefly luciferase TEAD reporter in combination with a control Renilla luciferase construct. Initial screens will be performed with a library comprising all compounds that have undergone Phase 1 trial for any indication (~4,000) and then with a library comprising a 'diversity' set of compounds (~17,600). Hits will be prioritized by using selective inhibition of proliferation of NF2-mutant cells as the major criterium (year 1).

Because of the transition to MDACC, we were not able to initiate the screens at the Rockefeller High Throughput Screening Facility, as we had anticipated in our Application. However, we have worked in collaboration with the newly established High Throughput Screening Facility of MSKCC along the lines described in Accomplishments below. Notably, this Facility is equipped for High Throughput Confocal Imaging and Analysis (6, 7), which will be instrumental for the execution of the screens in the near future.

Task 2. Subsequent screens will involve a large library comprising more than 178,000 small molecules. Hits will be prioritized by using selective inhibition of proliferation of NF2-mutant cells as the major criterium (year 2).

This screen will be now performed with independent funding.

Task 3. Cell biological and biochemical experiments will be conducted to determine if any of the lead compounds inhibits CRL4^{DCAF1} or YAP and by what mechanism (year 3).

These experiments will also be conducted with independent funding.

What was accomplished under these goals?

During the past year of this grant, I have moved my research program from Memorial Sloan Kettering Cancer Center in New York to UT MD Anderson Cancer Center in Houston. In spite of extensive planning and my general assessment that the transition was relatively smooth, there is no question that the lab move has had some impact on our productivity. This assessment extends to all of my projects, including the one object of this Report. This said, in the past year we have made significant inroads into understanding the mechanism by which Merlin suppresses tumorigenesis that will greatly facilitate the development of new therapies for NF2, which represents the ultimate long term goal of this grant.

Although our studies have identified a critical role for nuclear Merlin in inhibition of CRL4^{DCAF1} and hence stabilization of active Lats, several questions remain unanswered. In particular, which other signaling mechanisms drive the development of NF2 mutant tumors? And are these mechanisms activated by deregulated CRL4^{DCAF1} or by the loss of Merlin from the cell cortex? In this respect, it is of interest that Merlin interacts through its coiled-coil segment with the central coiled-coil segment of AMOT proteins, which localize to Adherens Junctions (AJs) and Tights Junctions (TJs) by binding to α -catenin and PatJ,

respectively (8, 9, 10). Merlin binding to AMOT has been implicated in suppression of Rac and activation of Lats kinase activity (10, 11, 12, 13). Relevant to the pathways implicated in sustaining the oncogenicity of NF2 mutant cells, we have been examining the mechanism through which loss of Merlin activates mTOR. Intriguingly, we found that Merlin suppresses activation of mTOR by activating AMPK - independently of LKB1 (not shown). Tandem Affinity Purification followed by mass spectrometry revealed that AMOTL1 interacts with both AMPK and TSC1, suggesting that the Merlin-AMOTL1 complex functions as a scaffold for AMPK-mediated activation of TSC1/2 and hence suppression of mTOR under conditions of nutrient deprivation. Immunoprecipitation experiments confirmed that AMOTL1 interacts with Merlin and TSC1, whereas AMOT p130 and p80 interact only with Merlin. Silencing of Merlin did not reduce the association of AMOTL1 with TSC1, potentially implicating the FERM domain of Merlin - its "business portion" - in the interaction with TSC1. The interaction of AMOTL1 with AMPK was also confirmed by immunoprecipitation. These results suggest that upon establishing a coiled-coil interaction with AMOTL1, Merlin deploys its FERM domain in order to regulate signaling through AMPK and TSC1/2. These findings provide a novel model for Merlin regulation of mTOR. Moreover, since mTOR and YAP signaling are interconnected and both exert a pro-tumorigenic function (14, 15, 16, 17), these results indicate that it will be important to conduct the high throughput screen in this grant in the presence of a non-growth inhibitory concentration of a TORC1/2 inhibitor. As we continue to elucidate the mechanisms through which Merlin suppresses tumorigenesis, we also obtain information useful to identify the molecular targets of the small molecules we identify from the screen that is object of this grant.

We generated several reporter-expressing cell lines and tested them. FC1801 and MB231 cells were stably transduced with a Firefly luciferase TEAD reporter in combination with

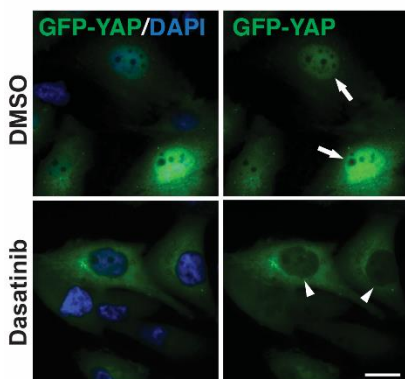


Fig. 1. Nuclear translocation of YAP is inhibited by Dasatinib treatment. MDA-MB-231 Cells stably expressing GFP-YAP treated with vehicle (DMSO) or Dasatinib (200 nM, 12 hours) were fixed and stained with DAPI. Arrows, cell with YAP nuclear translocation; Arrowheads, cells with YAP nuclear exclusion. Scale bar, 10 μ m.

a control Renilla luciferase construct and several clones of each derivative were isolated. TEAD-dependent expression of Firefly luciferase and background expression of Renilla luciferase was monitored by bioluminescence to identify clones expressing low levels of Renilla luciferase and high levels of Firefly luciferase. Control experiments were conducted to verify that expression from the TEAD reporter is inhibited by re-expression of Merlin, silencing of DCAF1, and overexpression of Lats1/2. Verteporfin, which inhibits the interaction of YAP with TEAD, was used as positive control. Analysis of the results, however, indicated that the TEAD reporter-expressing cells did not constitute a robust system for high through put screening.

During the past few months, we therefore generated FC1801 and MB231 cells stably transduced with GFP-YAP, selected clones expressing high levels of GFP-YAP through several cycles of expansion and FACS sorting, and verified that GFP-YAP accumulated, as anticipated, in

the nucleus of these cells (Fig. 1 top panels and not shown). Control experiments indicated that dasatinib, which blocks Src-mediated phosphorylation and inhibition of Lats (not shown), induces extrusion of GFP-YAP from the nucleus of MB231 cells (Fig. 1 bottom panels). Because of the strong activation of YAP in MB231 cells and the clarity of the effect of dasatinib, we decided to use MB231 cells expressing GFP-YAP for high through put screening. Essentially, we will use optical imaging to assess the effect of compounds in the high throughput screen on nuclear accumulation of YAP using dasatinib as positive control.

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

The postdoctoral fellow involved in this project has learned general principles of high throughput screening from Ralph Garippa, who heads the MSKCC High Throughput Screening Facility. Furthermore, she had the opportunity to discuss her project with me on a weekly basis and to attend to several seminars and work-in-progress meetings at MSKCC.

How were the results disseminated to communities of interest?

Nothing to report.

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

In the near future, we plan to conduct high throughput screens with a library comprising all compounds that have undergone Phase 1 trial for any indication (~4,000) and then with a library comprising a diversity set of compounds (~17,600). Hits will be prioritized by using selective inhibition of proliferation of NF2-mutant cells as the major criterium.

4. IMPACT:

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

We have made major inroads into understanding the multiple mechanisms through which Merlin suppresses tumorigenesis. Our results indicate that cortical Merlin combines with AMOTL1 to activate AMPK and hence suppress mTOR whereas nuclear Merlin inhibits CRL4^{DCAF1}-mediated activation of YAP. We have also developed NF2 mutant cell lines for the screens.

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 and problems are included above*

6. PRODUCTS: *Nothing to Report.*

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name:	<i>Filippo Giancotti, MD, PhD</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>0.30</i>
Contribution to Project:	<i>Principal Investigator during reporting period</i>
Funding Support:	<i>Extramural awards, institutional funds</i>
Name:	<i>Derek Tan, PhD</i>
Project Role:	<i>Co-investigator</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>0.30</i>
Contribution to Project:	<i>Co-investigator during reporting period</i>
Funding Support:	
Name:	<i>Young-Mi Kim</i>
Project Role:	<i>Research Associate</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>6</i>
Contribution to Project:	<i>Dr. Kim has performed experiments with the NF2 mutant NB231 cells.</i>
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Below are the changes in the active other support for Drs. Giancotti and Tan since the last reporting period:

Dr. Filippo Giancotti

1 R01CA191222-01A1 (PI: Giancotti /Zauderer) NCI	1/1/2016 - 12/31/2020 \$ 306,549	1.80 calendar
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Therapeutic efficacy of the CRL inhibitor MLN4924 in NF2 mutant mesothelioma
We propose to develop a therapeutic strategy based on inhibition of CRL4DCAF1 with MLN4924 in these highly malignant tumors.

Role: Principal Investigator

1 R35 CA197566-01 (PI: Giancotti) NCI Outstanding Investigator Award	8/1/2016 - 7/31/2023 \$ 600,000	6.00 calendar
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Mechanisms governing metastatic dormancy and reactivation
Having disseminated throughout the body and seeded pre-metastatic sites prior to surgery, cancer cells often remain dormant for several years. This Application seeks to elucidate the cellular circuits that sustain the survival and those that eventually trigger the reactivation of dormant cancer cells. Role:

Principal Investigator

RR160031 (PI: Giancotti) Cancer Prevention & Research Institute of Texas (CPRIT)	3/1/2016 - 2/28/2021 \$ 1,200,000	1.80 calendar
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Recruitment of Established Investigators Award

This award aims to recruit the world's best talent in cancer research by providing financial support to attract world class research scientists with distinguished professional careers to Texas universities and cancer research institutes.

Role: Principal Investigator

Dr. Derek Tan

R01AI118224-01A1 was awarded (12/01/2015 – 11/30/2020); 1.8 CM.

R01GM100477 is current in NCE. Dr. Tan's effort has been reduced to 1.5 CM via prior approval. Tri-Institutional Stem Cell Initiative **2013-034** (PI: Jaffrey) was completed on 12/31/2015.

P30CA008748-50 (PI: Thompson) has now added Dr. Tan as Co-Leader of the Experimental Therapeutics Program at 1.5 CM effort, no salary support, with a current project period through 12/31/2018.

R21AI122326 (MPI: Chakrabarti & Tan) was awarded (1/01/2016 - 12/31/2017); 0.6 CM effort

What other organizations were involved as partners?

Nothing to Report in addition to the transfer of the PI to MDACC.

8. SPECIAL REPORTING REQUIREMENTS: *None.*

9. APPENDICES:

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