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Award Number:
W81XWH-11-1-0043

TITLE: Regulation of Mammary Stem Cell Quiescence via Post-Translational Modification of
DeltaNp63alpha

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REPORT DATE: February 2014

TYPE OF REPORT:
Annual Summary

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				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE February 2014		2. REPORT TYPE Annual Summary		3. DATES COVERED 15 Nov 10 - 14 Nov 13	
4. TITLE AND SUBTITLE Regulation of Mammary Stem Cell Quiescence via Post-Translational Modification of DeltaNp63alpha				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-11-1-0043	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Andrew. J DeCastro, B.S., Pratima Cherukuri, M.S., James DiRenzo, PhD. E-Mail: Andrew.J.DeCastro@Dartmouth.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Dartmouth College Hanover, NH 03755				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 This document is the annual summary report for the training grant awarded to Andrew DeCastro entitled <i>Regulation of Mammary Stem Cell Quiescence via Post-Translational Modification of ΔNP63α</i> . Here, we report our findings under Task 3, as we have already completed our work on Tasks 1 and 2 in past years. Task 3 aims to determine the contribution of ΔNP63α and ΔNP63α-phosphorylation to therapeutic resistance in breast cancer stem cells. Based on or previous work, in which we show that TGF-β mediates ALK5 dependent phosphorylation of ΔNP63α, we also observed noticeable changes in cell phenotype indicative of EMT, a process utilized by cancer cells to mediate invasiveness, metastasis, chemoresistance and recurrence. As a result, we sought to determine the contribution of ΔNP63α and its phosphorylation to the process of EMT and demonstrated that ΔNP63α not only opposes TGF-β induced EMT, but was sufficient to reverse a post-EMT breast cancer cell line back towards a more epithelial phenotype (MET). The data presented here identifies a role for ΔNP63α as a potent oppose to TGF-β mediated EMT.					
15. SUBJECT TERMS Breast Cancer, stem cells, quiescence, phosphorylation, ΔNP63α, TGF-β					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	26	19b. TELEPHONE NUMBER (include area code)

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Introduction

The training grant entitled, “Regulation of Mammary Stem Cell Quiescence via Post-Translational Modification of ΔNp63α”, seeks to identify the kinase(s) responsible for the phosphorylation of ΔNp63α at serines 66 and 68 and to further characterize and understand the biological consequences of such phosphorylation. The purpose of the work funded by the above titled training grant aims to identify the particular function of serine 66/68 phosphorylation specifically on mammary stem cell regulation and phenotype and whether it may contribute to breast cancer progression, phenotype and/or recurrence. Completion of this work will further our understanding of the regulation and roles of ΔNp63α in the mammary gland and its contribution to breast tumorigenesis. Additionally, identification of the kinases responsible for such phosphorylation and its influence over ΔNp63α activity may make them favorable candidates for therapeutic targets.

Body

Task 1: Determine the effects of wild-type, phosphor-ablative and phosphor-mimetic alleles of ΔNp63α phosphorylation on stem cell behavior *in vitro*.

For this particular aim, we sought to explore the role and regulation of ΔNp63α in adult mammary stem cells resultant of phosphorylation at serines 66 and 68. In previous annual summary reports we have characterized such functions and consequently completed our work for this task. Briefly, we identified a relationship between phosphor-p63 expression and a quiescent-like state as observed via reduced rates of proliferation and accumulation of cells in G0/G1 of the cell cycle. Following a screening assay to identify the kinase(s) responsible, our top candidate was TGFβR1, also known as ALK5 (Task 2), for which we began to characterize the function of this kinase on phosphorylation of ΔNp63α and its downstream activity, which we characterize and complete in task 2 of the training grant (1)(Appendix 1- annual summary report:2012 – containing publication).

Task 2: Identify putative ΔNp63α-kinases and determine their role in mitotic activation of ΔNp63α-positive cells.

In the previous annual summary report we had completed task 2, resulting in a publication, all of which can be seen in appendix 1 (Annual summary report – 2012 with publication). Briefly, we determined that upon stimulation with TGF-β, the intracellular domain of the ALK5 kinase is cleaved and translocates into the nucleus where it is able to phosphorylate ΔNp63α. ALK5 mediated phosphorylation of ΔNp63α resulted in an accumulation of cells in G0/G1 phase of the cell cycle, indicating an anti-proliferative phenotype (Appendix 1: Figure 5B). Taken together, we concluded that phosphorylation of ΔNp63α by ALK5 was a destabilizing, anti-proliferative and anti-clonogenic event (Appendix 1).

Task 3: Determine the contribution of ΔNp63α and ΔNp63α-phosphorylation to therapeutic resistance in breast cancer stem cells.

During our experiments to further characterize the function of ALK5-mediated phosphorylation of ΔNp63α (Tasks 1 & 2), we observed significant morphological changes in cells treated in the presence of TGF-β (500 pM), the ligand of ALK5 (Figure 1A). These changes were indicative of an epithelial-to-mesenchymal transition (EMT). Moreover, this observed EMT in response to TGF-β was inhibited via co-treatment with the ALK5 small molecular inhibitor A83-01 (2 μM) as indicated via morphological phenotype and immunofluorescent staining against E-cadherin, a marker of epithelial cells (Figure 1A). To further characterize this observed morphological change in response to TGF-β as EMT we performed a

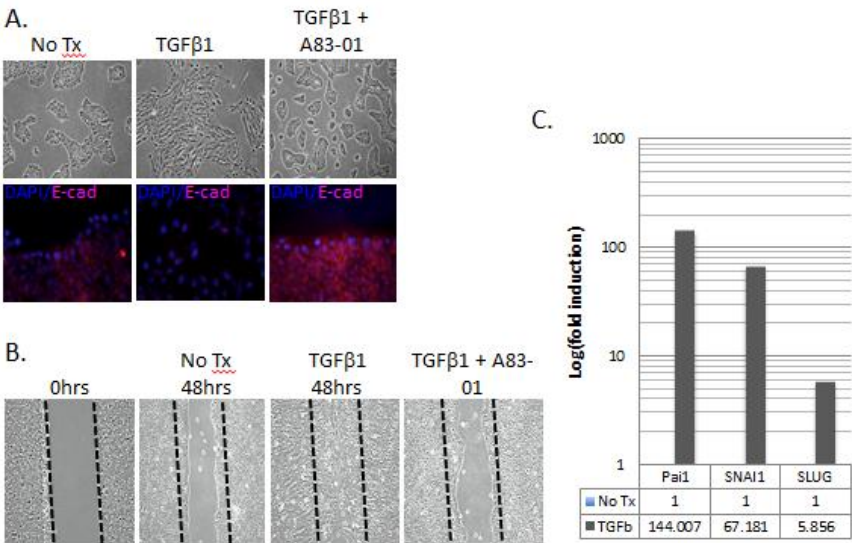


Figure 1: TGF-β stimulates EMT in MCF10A cells

scratch assay, a measure of the invasive properties of EMT cells (Figure 1B). Cells were plated at near confluent density and were subsequently treated with either no treatment control, TGF- β (500 pM) or TGF- β (500 pM) and A83-01 (2 μ M). A scratch was then made down the middle of the plate, and re-colonization of the scratch was measured 48 hours after the scratch to measure the invasive properties. As indicated via the images (Figure 1B), TGF- β greatly increased the invasive properties of MCF10A cells, and this was significantly diminished via co-treatment with A83-01. Likewise, EMT was further validated in TGF- β treated cells via qRT-PCR analysis revealing a significant relative induction of EMT associated genes *Pai1*, *Snai1* and *Slug* (Figure 1C). These results are significant as EMT is a key developmental program that can be reactivated upon tumorigenesis and has been linked to tumor invasion, metastasis, chemoresistance and recurrence. Additionally, cancer cells that have undergone EMT acquire a cancer stem cell phenotype (2-4). This, taken together with our results demonstrating TGF- β mediated ALK5 phosphorylation of Δ Np63 α raised the question of whether Δ Np63 α may oppose EMT.

To answer this, we first analyzed the morphology of cells in response to TGF- β treatment in the presence or absence of constitutive Δ Np63 α expression (Figure 2A). MCF10A cells were seeded onto a plate at sub-confluence and then treated with either TGF- β (50 pM), A83-01 (2 μ M), an adenovirus to overexpress Δ Np63 α or Δ Np63 α overexpression and TGF- β . Three days post treatments, compared to vehicle, which are highly epithelial in structure; TGF- β treated cells had undergone an EMT, as indicated via less organized and less tight junctions and less rounded all features of mesenchymal cells. Conversely, ectopic expression of Δ Np63 α garnered cells with a more rounded epithelial phenotype, which remained relatively unperturbed via the introduction of TGF- β (Figure 2A), suggesting that Δ Np63 α opposes TGF- β induced EMT.

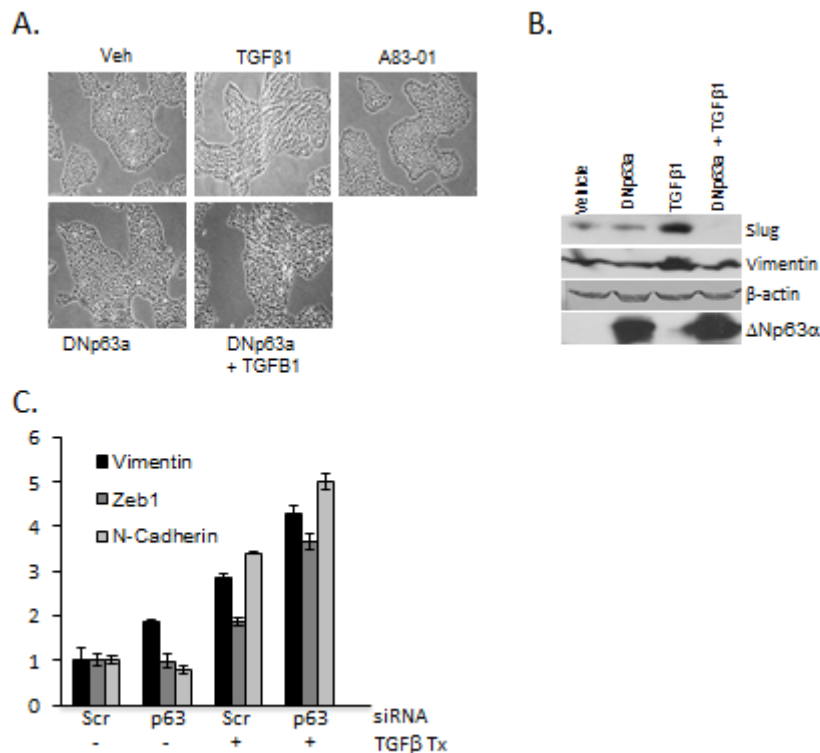


Figure 2: Δ NP63 α opposes TGF- β mediated EMT in MCF10A cells

Likewise, when we analyzed the expression of Slug and Vimentin, two proteins associated with EMT via western blot, we saw that Δ Np63 α was able to prevent the observed induction of these proteins in response to TGF- β (Figure 2B), further validating Δ Np63 α 's role in opposing EMT. Finally, we analyzed the mRNA expression of multiple genes implicated in EMT (Vimentin, ZEB1 and N-Cadherin) in response to siRNA mediated knockdown of endogenous Δ Np63 α in the presence or absence of the EMT inducing molecule TGF- β (Figure 2C). To do this we first transfected cells with either scramble or siRNA directed against Δ Np63 α (20nM). Twenty four hours post transfection, cells were then treated with TGF- β (50pM) and subsequently collected for qRT-PCR analysis 24 hours later. In response to siRNA-knockdown of Δ Np63 α , compared to scramble control, there was an induction of mRNA of the pro-EMT gene Vimentin, however, there was little to no change in the expression of the other two pro-EMT genes analyzed. As expected, in response to TGF- β alone, there was a large induction of all three pro-EMT genes mRNA expression. Interestingly, this was further increased in response to siRNA mediated ablation of Δ Np63 α , further suggesting that Δ Np63 α opposes TGF- β mediated EMT (Figure 2C).

Finally, we wanted to determine if $\Delta Np63\alpha$ was sufficient to induce an MET in a cell line that is phenotypically in an EMT state. For this, the MDA-MB-231 breast cancer cell line was used, which are post-EMT and display highly aggressive and invasive characteristics that are hallmarks of EMT. Additionally, MDA-MB-231 cells do not express detectable levels of $\Delta Np63\alpha$. Upon adenoviral overexpression of $\Delta Np63\alpha$, MDA-MB-231 cells appeared more epithelial in structure and had highly organized tight junctions compared to adenoviral control (Figure 3A). Likewise, when we performed scratch assays, similar to that described above, we observed a significant reduction in MDA-MB-231 cells to migrate into the cleared area in the presence of $\Delta Np63\alpha$, further illustrating $\Delta Np63\alpha$'s potent anti-EMT, pro-MET effects (Figure 3B). Additionally, when we treated MDA-MB-231 cells with either $\Delta Np63\alpha$ or the ALK5 inhibitor A83-01, there was an observed reduction in the mRNA expression of the three EMT markers Vimentin, SLUG and ZEB1 (Figure 3C). Both $\Delta Np63\alpha$ and A83-01 treatment phenocopy each other, which is supported by our previous finding showing that TGF- β mediated ALK5 phosphorylation of $\Delta Np63\alpha$ is a destabilizing event of $\Delta Np63\alpha$, therefore enabling TGF- β to promote EMT. However, in the presence of this inhibitor (A83-01), phosphorylation of $\Delta Np63\alpha$ is prevented, thereby enabling $\Delta Np63\alpha$ to oppose EMT and promote MET. Finally, to further validate $\Delta Np63\alpha$'s role in mediating MET, western blot analysis for cytokeratin's indicative of an epithelial phenotype (cytokeratin's 14 & 17) were performed in MDA-MB-231 cells treated with vehicle and adenoviral control, or an adenovirus expressing $\Delta Np63\alpha$, or A83-01 (Figure 3D). Results show that $\Delta Np63\alpha$ is sufficient to induce expression of cytokeratin's 14 and 17, which is consistent with the induction of an epithelial phenotype Figure 3D).

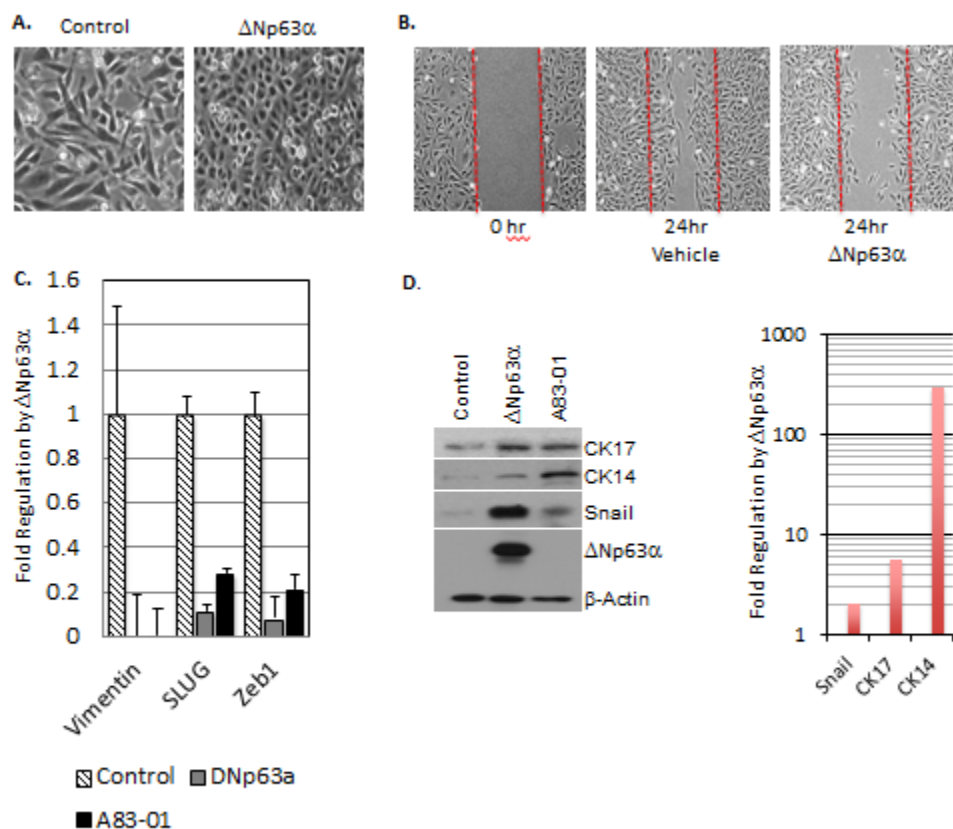


Figure 3: $\Delta Np63\alpha$ is sufficient to convert MDA-MB-231 cells from an EMT state to MET

Key Research Accomplishments

- Determined that TGF- β induces EMT in the breast cell line MCF10A.
- Determined that ectopic $\Delta Np63\alpha$ was able to block TGF- β mediated induction of EMT in MCF10A cells.
- Determined that $\Delta Np63\alpha$ is sufficient to convert a post-EMT breast cancer cell line (MDA-MB-231) to an epithelial phenotype via MET.

Reportable Outcomes

- There are no reportable outcomes at this time. However, we intend to submit a manuscript, of which the above reported data is part of within the near future.

Conclusions

To date, we have shown that Δ Np63 α is able to oppose TGF- β mediated EMT of normal and breast cancer cells. Additionally we show that Δ Np63 α is able to revert post-EMT cells back towards an epithelial phenotype. Moreover, based on the observations that ALK5 inhibition via A83-01 was able to phenocopy the effects of Δ Np63 α overexpression with result to MET, it suggests that TGF- β may be mediating its pro-EMT effects through activation and subsequent phosphorylation of Δ Np63 α by ALK5, which we have previously shown is a destabilizing event. Collectively, the data suggests that Δ Np63 α is present to oppose EMT, which is a process utilized by breast cancer to promote invasiveness, metastasis and overall poor prognosis in cancer patients. This data also brings to light the possible utility of using an ALK5-inhibitor in the clinic to prevent or reverse the process of EMT and its subsequent regulation over tumor phenotype.

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Appendices

Appendix 1:

W81XWH-11-1-0043 Annual Summary Report 2012 containing the following publication:

Cherukuri, P., **DeCastro, A.**, Balboni, A., Downey, S., Liu, J., Hutchinson, J., D'Amico, J. (2012). Phosphorylation of Δ Np63 α via a Novel TGF β /ALK5 Signaling Mechanism Mediates the Anti-Clonogenic Effects of TGF β . *PLoS1*, 7(11); e50066.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 14-12-2012		2. REPORT TYPE Annual Summary Report		3. DATES COVERED 01JAN2012 – 14DEC-2012	
4. TITLE AND SUBTITLE Regulation of Mammary Stem Cell Quiescence via Post-translational Modification of Δ NP63 α				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-11-1-0043	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Andrew. J DeCastro, B.S., Pratima Cherukuri, M.S., James DiRenzo, PhD. E-Mail: Andrew.J.DeCastro@Dartmouth.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Dartmouth College, Hanover, NH 03755				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 This document is the Annual Summary Report on the training grant awarded to Andrew DeCastro entitled <i>Regulation of Mammary Stem Cell Quiescence via Post-translational Modification of ΔNP63α</i> . Here, we report our findings of the effects of TGF β (previously validated as a kinase in our kinome screen) mediated phosphorylation of Δ NP63 α on stem cell behavior and mitotic activity. Task 1 aims to determine the effects of wild-type, phospho-ablative and phospho-mimetic alleles of Δ NP63 α phosphorylation on stem cell behavior <i>in vitro</i> . Thus far, we demonstrate that stem cell enriched populations, as indicated by ALDH1 activity, contain higher concentrations of Δ NP63 α mRNA. In addition, we demonstrate that the anti-clonogenic effects of TGF β are mediated through phosphorylation of Δ NP63 α , which is rescued via ectopic expression of the phospho-ablative mutant Δ NP63 α -AA. Task 2 aims to identify putative Δ NP63 α -kinases and determine their role in mitotic activation. Here we further characterize TGF β -mediated phosphorylation of Δ NP63 α . We demonstrate that TGF β phosphorylation of Δ NP63 α is a destabilizing event, and dependent on the proteasomal degradation pathway. We also report a non-canonical pathway in which ALK5 (TGF β R1) is cleaved from the membrane and translocates to the nucleus, where it phosphorylates Δ NP63 α . Due to our focus on Task 1 and 2, we have not yet begun our investigation of Aim 3.					
15. SUBJECT TERMS Breast Cancer, stem cells, quiescence, phosphorylation, Δ NP63 α , TGF- β					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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AD _____

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PRINCIPAL INVESTIGATOR:
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CONTRACTING ORGANIZATION:
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REPORT DATE:
December 14th, 2012

TYPE OF REPORT:
Annual Summary Report

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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Introduction

The training grant entitled, "Regulation of Mammary Stem Cell Quiescence via Post-translational Modification of Δ NP63 α ", aims to identify the kinase(s) responsible for phosphorylation of Δ NP63 α at serines 66 and 68 and to characterize the function of this phosphorylation. More specifically, the purpose of this research is to characterize the contribution of this phosphorylation on stem cell and mitotic activity, and whether it plays a role in breast cancer recurrence. Completion of this research will further add to our understanding of the role and regulation Δ NP63 α in mammary and cancer stem cells, and identify kinases as therapeutic targets to minimize or reduce breast cancer recurrence, a significant contributor of breast cancer mortality.

Body

Task 1: Determine the effects of wild-type, phospho-ablative and phospho-mimetic alleles of Δ NP63 α phosphorylation on stem cell behavior *in vitro*.

Δ NP63 α has been shown to play a critical role in maintaining replicative capacity of adult epithelial stem cells, and because many mechanisms of normal stem cell function are utilized in cancer stem cells to garner therapeutic resistance and recurrence(1-3), we sought to explore the role and regulation of Δ NP63 α in adult mammary stem cells. In the past annual summary report, we identified a relationship between phospho-p63 expression and a quiescent state, which is a feature often utilized by mammary stem cells to maintain replicative potential. Additionally, we also performed a kinome screening assay to determine the kinase(s) responsible for this phosphorylation and identified TGF β R1 (ALK5) as a kinase. In this report, we characterize the function of TGF β -mediated phosphorylation of Δ NP63 α on stem cell behavior. To do this, we treated immortalized mammary epithelial cells (IMECs) with vehicle control, TGF β or TGF β in the presence of A83-01 (small molecule inhibitor of ALK5) up to 72 hours and measured cell number. Results indicate that TGF β significantly reduced cell growth and was rescued via inhibition of TGF β by A83-01 (Appendix 1, figure 5A). This coincided with an increase of cells in G0/G1 compared to vehicle control, and was rescued with addition of A83-01 (Appendix 1: Figure 5B). Together, these results demonstrate that TGF β has an anti-proliferative effect in IMECs, which is a common feature of stem cells. When IMECs were FACS sorted based on ALDH activity (a marker of stem cells) and analyzed for Δ NP63 α mRNA expression, ALDH^{high} fractions, which are enriched for stem cells, had significantly higher levels of Δ NP63 α (Appendix 1: Figure 5C). ALDH activity was then assessed in cells treated with vehicle, TGF β or A83-01. TGF β treated cells had reduced ALDH activity compared to controls which was rescued via A83-01 treatment (Appendix 1: Figure 5D). Collectively, these results identify Δ NP63 α to be highly expressed in stem cell fractions, which are reduced via addition of TGF β , which activates a kinase we validated that phosphorylates Δ NP63 α . In addition, we show that TGF β mediated phosphorylation of Δ NP63 α is a destabilizing event (Appendix: Figure 6). Taken together, our results suggest that the TGF β -mediated reduction of ALDH activity, a marker of stem cells, is mediated through phosphorylation and subsequent reduction of Δ NP63 α protein, a known regulator of stem cell maintenance. Furthermore, we demonstrate that the anti-clonogenic activity of TGF β is mediated, in part, through phosphorylation of Δ NP63 α , which was rescued via ectopic expression of the phospho-ablative mutant Δ NP63 α -AA (Appendix1: Figure 7). Thus far, this series of experiments represent our findings for this task for the given period of the report, furthermore, they are part of an original research paper published in the November 2012, volume 7, issue 11 of PLoS1 Journal (Appendix 1).

Task 2: Identify putative Δ NP63 α -kinases and determine their role in mitotic activation of Δ NP63 α -positive cells.

In the previous annual summary report, we identified putative kinases that phosphorylate Δ NP63 α including TGF β R1 (ALK5). Due to its significance in cell and cancer biology (4, 5) we validated and confirmed TGF β R1 as a kinase of Δ NP63 α using three different siRNAs against the receptor. Since then, we have characterized the mechanisms and the functions of TGF β R1-mediated phosphorylation of Δ NP63 α which have been published in an original paper in PLoS1 Journal (Appendix 1). To determine if TGF β R1 (ALK5) directly phosphorylates Δ NP63 α , we performed an *in vitro* kinase assay in which affinity purified ALK5 and Δ NP63 α were incubated together at varying concentrations. The protocol for this assay is detailed in the materials and methods section of the publication (Appendix 1). Western blot analysis for phospho-p63 demonstrated that ALK5 directly phosphorylate Δ NP63 α (Appendix1: Figure 1D). We then sought to determine if the ligand (TGF β) for this kinase (ALK5) was sufficient to phosphorylate Δ NP63 α . Western blot analysis resulted in increased phospho- Δ NP63 α levels in TGF β treated cells (Appendix1: Figure 2A). To determine if this TGF β mediated phosphorylation required ALK5 activity, we performed the same assay in the presence of a small molecule inhibitor (A83-01) against ALK5. Western blot analysis revealed that ALK5 is indeed necessary for TGF β -mediated phosphorylation of Δ NP63 α (Appendix1: Figure 2A). However, because ALK5 possesses no TGF β binding capacity, we sought to determine

if phosphorylation of Δ NP63 α requires TGF β R2, which does bind TGF β . Western blot analysis of cells co-transfected with/without an siRNA against TGF β R2 and with/without Δ NP63 α revealed that this phosphorylation event requires TGF β R2 and that phosphorylation of Δ NP63 α is facilitated via the association of ALK5 and TGF β R2, which is mediated through TGF β ligand stimulation (Appendix1: Figure 2C and 2D respectively).

Next, we examined the mechanism by which a cell surface kinase (ALK5) can phosphorylate Δ NP63 α , a nuclear protein. Western blot analysis of IMECs treated with siRNA against ALK5 revealed a 34kDa fragment that coincided with the intracellular region of ALK5 (Appendix1: Figure 6A). Previous studies have made evident that the intracellular region of ALK5 is sensitive to proteolytic cleavage where it then accumulates in the nucleus (6). To determine if this observed intracellular fragment of ALK5 localizes to the nucleus, we isolated nuclear and cytoplasmic lysates from IMECs, which were then analyzed via western blot to detect the localization of cleaved ALK5. We observed accumulation of the cleaved fragment in the nucleus (Appendix1: Figure 6C). Additionally, when we transfected an ALK5-GFP fusion expression vector into H1299 cells and subsequently treated with vehicle or TGF β , we were able to show that the fusion protein localizes to the nucleus (Appendix1: Figure 6D). This nuclear accumulation was also found to occur with endogenous ALK5 in response to TGF β stimulation (Appendix1: Figure 6E). Taken together, we demonstrate that Δ NP63 α is phosphorylated in response to TGF β through cleavage of the intracellular ALK5 domain, which subsequently translocates into the nucleus.

Thus far, we have further characterized the mechanism(s) of ALK5 mediated phosphorylation of Δ NP63 α . We determined that this phosphorylation is direct, is in response to TGF β ligand stimulation and requires TGF β R2. Furthermore, we demonstrate that the intracellular domain of ALK5 translocates into the nucleus to phosphorylate Δ NP63 α . We are currently in the process of further characterizing the biological significance of the observed TGF β mediated phosphorylation of Δ NP63 α with respect to mitotic and stem cell activity.

Task 3: Determine the contribution of Δ NP63 α and Δ NP63 α -phosphorylation to therapeutic resistance in breast cancer stem cells.

Due to our heavy focus on Task 1 and 2, we have only recently begun work focusing on Task 3. Previous publications have made evident that Δ NP63 α is phosphorylated upon UV-irradiation (7). We sought to determine if this phosphorylation is mediated through the TGF β -ALK5 signaling pathway. To do this, we plated IMECs and treated them with mock or UV-irradiation followed by vehicle or A83-01 (small molecular inhibitor of ALK5). Western blot analysis revealed inhibition of UV-induced phosphorylation of Δ NP63 α when ALK5 was inhibited, indicating that UV-induced phosphorylation is mediated through the ALK5 pathway (Appendix 1: Figure 3A). Additionally, these results made evident that in response to UV-irradiation, TGF β signaling was induced, which was indicated via increased phospho-Smad2 levels (Appendix 1: Figure 3A). Similar experiments in which we transfect H1299 cells with wild type Δ NP63 α or the phospho-ablative mutant Δ NP63 α -AA, followed by UV-irradiation and A83-01 treatment demonstrated that UV-induced phosphorylation of Δ NP63 α is mediated through induction of TGF β and ALK5 signaling (Appendix 1: Figure 3B). These results are relevant to Task 3 as UV-irradiation initiates cellular stress and pro-survival responses; of which Δ NP63 α has been shown to play a role in HNSCC (8) and triple negative breast cancers chemosensitivity (9).

Key Research Accomplishments

- Determined that TGF β 's anti-clonogenic effects are mediated through ALK5-mediated phosphorylation of Δ NP63 α .
- Δ NP63 α is enriched in stem cells as indicated by ALDH activity and TGF β treatment decreases the stem cell population in an ALK5 dependent manner.
- TGF β -mediated phosphorylation of Δ NP63 α is a destabilizing event.
- ALK5 directly phosphorylates Δ NP63 α .
- ALK5-mediated phosphorylation of Δ NP63 α requires TGF β R2.
- ALK5 intracellular fragment localizes to the nucleus to phosphorylate Δ NP63 α in response to TGF β stimulation.
- UV-induced phosphorylation of Δ NP63 α is mediated through ALK5 signaling.

Reportable Outcomes

- Original paper publication:
Cherukuri, P., **DeCastro, A.**, Balboni, A., Downey, S., Liu, J., Hutchinson, J., Drenzo, J. (2012). Phosphorylation of Δ NP63 α via a Novel TGF β /ALK5 Signaling Mechanism Mediates the Anti-Clonogenic Effects of TGF β . PLoS1, 7(11); e50066. (Publication included as Appendix 1).

Conclusion

To date, we have elucidated the mechanism by which TGF β /ALK5 phosphorylates Δ NP63 α . We have shown that this phosphorylation event is direct, and involves nuclear translocation of the intracellular portion of ALK5. Furthermore, we have demonstrated that TGF β -mediated phosphorylation of Δ NP63 α has significant effects on the stem cell activity (ALDH^{Hi}), and is anti-clonogenic and causes an increase in G0/G1 cell cycle arrest. These results are significant as they describe a novel signaling pathway of TGF β that involves Δ NP63 α . Additionally, the anti-clonogenic and anti-stem cell effects of TGF β , which are mediated via Δ NP63 α , a destabilizing events, implicates Δ NP63 α as a pro-tumorigenic factor, and brings to light a potential therapeutic strategy utilizing TGF β to reduce Δ NP63 α expression. As a result, we will continue to investigate the function of Δ NP63 α phosphorylation as outlined in the tasks of the Approved Statement of Work.

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Appendices

Appendix 1:

Cherukuri, P., **DeCastro, A.**, Balboni, A., Downey, S., Liu, J., Hutchinson, J., Drenzo, J. (2012). Phosphorylation of Δ NP63 α via a Novel TGF β /ALK5 Signaling Mechanism Mediates the Anti-Clonogenic Effects of TGF β . PLoS1, 7(11); e50066.

Phosphorylation of Δ Np63 α via a Novel TGF β /ALK5 Signaling Mechanism Mediates the Anti-Clonogenic Effects of TGF β

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Abstract

Genetic analysis of *TP63* implicates Δ Np63 isoforms in preservation of replicative capacity and cellular lifespan within adult stem cells. Δ Np63 α is also an oncogene and survival factor that mediates therapeutic resistance in squamous carcinomas. These diverse activities are the result of genetic and functional interactions between TP63 and an array of morphogenic and morphostatic signals that govern tissue and tumor stasis, mitotic polarity, and cell fate; however the cellular signals that account for specific functions of *TP63* are incompletely understood. To address this we sought to identify signaling pathways that regulate expression, stability or activity of Δ Np63 α . An siRNA-based screen of the human kinome identified the Type 1 TGF β receptor, ALK5, as the kinase required for phosphorylation of Δ Np63 α at Serine 66/68 (S66/68). This activity is TGF β -dependent and sensitive to either ALK5-directed siRNA or the ALK5 kinase inhibitor A83-01. Mechanistic studies support a model in which ALK5 is proteolytically cleaved at the internal juxtamembrane region resulting in the translocation of the C-terminal ALK5-intracellular kinase domain (ALK5^{IKD}). In this study, we demonstrate that ALK5-mediated phosphorylation of Δ Np63 α is required for the anti-clonogenic effects of TGF β and ectopic expression of ALK5^{IKD} mimics these effects. Finally, we present evidence that ultraviolet irradiation-mediated phosphorylation of Δ Np63 α is sensitive to ALK5 inhibitors. These findings identify a non-canonical TGF β -signaling pathway that mediates the anti-clonogenic effects of TGF β and the effects of cellular stress via Δ Np63 α phosphorylation.

Citation: Cherukuri P, DeCastro AJ, Balboni AL, Downey SL, Liu JY, et al. (2012) Phosphorylation of Δ Np63 α via a Novel TGF β /ALK5 Signaling Mechanism Mediates the Anti-Clonogenic Effects of TGF β . PLoS ONE 7(11): e50066. doi:10.1371/journal.pone.0050066

Editor: Carol Feghali-Bostwick, University of Pittsburgh, United States of America

Received: July 12, 2012; **Accepted:** October 19, 2012; **Published:** November 16, 2012

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Funding: This work was supported in part grants to J.D.R. from the National Cancer Institute (5R01CA108539-05), the United States Department of Defense Breast Cancer Research Program (BC095560) and a Prouty Pilot Award from the Friends of the Norris Cotton Cancer Center. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

TP63 is a member of the p53 family of transcriptional regulators [1] that preserves long-term regenerative stasis in diverse epithelial structures by maintaining the replicative capacity of adult stem cells [2,3]. Several lines of evidence also implicate TP63 in multiple aspects of cancer initiation and progression. The mechanisms by which TP63 carries out these critical functions in development and disease are not fully understood, and progress toward this end is complicated by the fact that TP63 encodes as many as eight p63 isoforms. Differential usage of distal and proximal promoters results in isoforms with (TAp63) or without (Δ Np63) an amino-terminal trans-activation domain homologous to that of p53. Additionally alternative mRNA splicing results in C-terminal diversity. Δ Np63 α is the predominant TP63 isoform in regenerative compartments of diverse epithelial structures and tumors of squamous epithelial origin. Isoform specific knockouts unambiguously indicate that the Δ Np63 isoforms account for the maintenance of replicative capacity [4,5]. A second layer of complexity arises from studies indicating that Δ Np63 α occupies greater than 5000 sites across the human genome and that these

sites correlate with activation and repression of transcriptional targets [6]. Finally, the stability, transcriptional activity and cellular localization of TP63 gene products are regulated post-translationally by multiple phosphorylation events as well as by ubiquitination [7], SUMOylation [8] and ISGylation [9]. This combination of isoform diversity, widespread genomic occupancy, and post-translational regulation underscores the challenges of identifying the regulatory mechanisms and transcriptional targets of TP63 that account for its complex role in tissue and tumor stasis.

Δ Np63 α has been shown to play important roles in cancer initiation and progression suggesting that pharmacologic strategies that disrupt the activity of Δ Np63 α have the potential for therapeutic benefit. Δ Np63 α is an oncogene that suppresses the activity of the Ink4A/ARF locus [10] and opposes the tumor suppressive effects of cellular senescence [11,12] suggesting a role in oncogenic initiation [13]. TP63 is amplified at the genomic level in 9.7% of head and neck squamous cell carcinomas, 12.9% of serous ovarian carcinomas, 23% of squamous cervical carcinomas and 28.5% of lung squamous cell carcinomas (<http://cbiportal>).

well at a concentration of 0.5 pmoles using siPORT NeoFX Transfection Agent (Cat#AM4511). Forty-eight hours post siRNA transfection; cells were infected with Δ Np63 α -WT adenovirus resulting in 90% infection efficiency. Quantitative immunofluorescence assays were then performed using the anti-phospho-p63 (Ser160/162) antibody. Immunofluorescence assays were performed as described below. Fluorescent values were obtained using the Molecular Devices Gemini XS Fluorescent Microplate Reader (EX: 540, EM: 570 and Cutoff: 570). Raw fluorescence values were normalized against total cell count, three different negative controls and a positive control. To obtain total cell count, cells after IF readings were stained with crystal violet. After a rigorous wash with distilled water, stain from the cells were eluted in a 20% methanol and 10% acetic acid solution, values were read at 595 nm using the Molecular Devices Thermo Max Microplate reader at 595 nm.

Small-interfering RNA (siRNA) Transfection

Silencer select pre validated small-interfering RNA against TGF β RI and TGF β RII (Catalog #: 4390824) were purchased from Ambion. Ambion's siPORT NeoFX Transfection Agent was used in all siRNA transfections according to the manufacturer's protocol. Ambion Silencer[®]Negative Control #2 siRNA (Cat#AM4613) was used as control for all siRNA transfections. Forty eight hours post siRNA transfection, cells were transfected with GFP, Δ Np63 α -WT or Δ Np63 α -AA mutant plasmids, and harvested 24 hrs after plasmid addition in 1XSDS lysis buffer for further analysis by immunoblotting.

Western Analysis and Immunofluorescence

Cells were lysed in 1XSDS lysis buffer with β -mercaptoethanol and were resolved by 10% SDS- PAGE. Antibodies used for western and Immunofluorescence were anti-p63 Clone 4A4 (Sigma, Cat#P3737) for total P63, anti-phospho-p63 (Ser160/162) (Cell signaling, Cat#4981), anti-phospho-Smad2 (Ser465/467) (Cell signaling, Cat#3108), anti-Smad2 (Cell signaling, Cat#3122), anti-TGF β RII (L-21) (Santa Cruz, Cat#sc-400), anti-TGF β RI (V-22) antibody from (Santa Cruz, Cat#sc-398) and anti- β -Actin antibody from (Cell signaling, Cat#3700). All primary antibodies were detected with their respective secondary HRP- conjugated antibodies using Millipore chemiluminescence. Primary antibodies for Immunofluorescence assays were detected with either anti-mouse-Alexa Fluor 488 (Invitrogen#A11029) or anti-rabbit-Alexa Fluor 555 (Invitrogen#A21429).

Colony Formation Assay

IMEC cells were plated at 1000 cells per well in a 6-well dish. Cells were fed every other day with TGF β 1 $\pm$ A83-01 for 10–14 days. Cells transfected with p63 constructs were selected for in the presence of G418. Colonies were fixed in ice-cold 80% methanol and stained with 0.5% crystal violet.

In vitro Kinase Assay

Recombinant ALK5 kinase was produced by transfecting H1299 cells with an expression vector programmed to produce C-terminally flag-tagged ALK5 Receptor. Immunoprecipitation of the Flag-tagged ALK5 kinase was performed using Pierce Classic IP kit and monoclonal ANTI-FLAG M2 antibody (Sigma Cat # F1804). Recombinant ALK5 was eluted from beads by incubation with 100 μ g/ml of soluble flag peptide (Sigma Cat # F3290) in 10 mM Tris, 150 mM NaCl, pH7.4 buffer. GST- Δ Np63 α was produced via IPTG induced expression in Y1090 cells. Bacteria were collected by centrifugation and lysed by sonication in the

presence of a cocktail of protease inhibitors. Lysates were cleared by centrifugation and GST- Δ Np63 α was enriched on glutathione-sepharose beads. For the kinase reaction either 1 or 5 μ L of soluble ALK5 was added to fixed amounts of GST- Δ Np63 α bound to beads in 1X kinase buffer (50 mM HEPES, 5 mM MgCl₂, 1 mM CaCl₂) with 10 μ M ATP. Reactions were incubated at 30°C for 30 min. Reactions were stopped by the addition of 2 $\times$ SDS Sample Buffer and western blots were done as described previously.

Aldefluor Assay

ALDH^{high} and ALDH^{low} populations in IMEC cells were identified using the Aldefluor assay kit (Stem Cell Technologies). IMEC cells were plated at 25% confluence and treated with Vehicle, TGF β 1 or A83-01 for 24 and 48 hours. Cells were then harvested and stained with ALDEFLOUR reagent as per the manufacturers protocol.

Cell Cycle Analysis and Subcellular Fractionation

Cells were collected by trypsinization and gentle centrifugation before being re-suspended in ice cold PBS. An equal volume of ice cold 80% methanol was added with gentle vortexing and cells were fixed on ice for 30 minutes. Fixed cells were collected by centrifugation and re-suspended in PBS supplemented with 0.5 μ g/ml of RNase A. After 45 minutes at 37°C, cells were stained with propidium iodide and samples were analyzed on a BD FACScan instrument. Nuclear and cytoplasmic extracts were prepared using the EpiQuik[™] Nuclear Extraction Kit I according to the manufacturer's protocol.

Results

A Small Interfering RNA Screen of the Human Kinome Identifies ALK5 as a Putative Δ Np63 α Kinase

To identify the signaling pathways governing the diverse activities of Δ Np63 α a siRNA-based screen of the human kinome was carried out in H1299 lung adenocarcinoma cells (Figure S1). H1299 cells do not express Δ Np63 α but rapidly phosphorylate ectopic wild-type Δ Np63 α (Δ Np63 α -WT) but not a mutant allele in which serines at positions 66 and 68 were changed to alanine (Δ Np63 α -AA) (Figure 1A). Pools of three kinase-specific siRNAs were transfected into H1299 cells. At 48 hours post transfection cells were infected with an adenovirus programmed to express Δ Np63 α . Twenty four hours post infection, phospho-p63 levels were measured by immunofluorescence and quantitated using a fluorescent plate reader. Following this analysis, cells were stained with crystal violet to record cell density. Analysis of phospho- Δ Np63 α immunofluorescence intensity normalized to cellular density (Figure 1B) resulted in the identification of several kinases (Table in Figure S1) with normalized phospho- Δ Np63 α scores lower than the negative control (red dashed line). Among these was the Type 1 TGF β Receptor (ALK5) and transfection of each of the three individual ALK5-directed siRNAs was sufficient to suppress Δ Np63 α phosphorylation at S66/68, indicating that ALK5 was necessary for Δ Np63 α phosphorylation (Figure 1C). To determine if ALK5 was sufficient to phosphorylate Δ Np63 α H1299 cells were transfected flag-tagged ALK5 and recombinant ALK5 was isolated by anti-flag affinity chromatography and eluted with soluble flag-peptide. One and five μ L of the eluted fraction was incubated with increasing amounts of bacterially expressed glutathione-S-transferase- Δ Np63 α fusion protein. Western analysis revealed that recombinant ALK5 was able to phosphorylate Δ Np63 α in vitro (Figure 1D). Given that both ALK5 and GST- Δ Np63 α were affinity purified, these data support the assertion

that ALK5 directly phosphorylates Δ Np63 α . This in turn raises questions regarding the mechanisms by which a membrane-bound kinase can phosphorylate a nuclear protein. Finally, H1299 cells were co-transfected with Δ Np63 α and a series of ALK5 expression plasmids that express wild type or mutant ALK5. Phospho-p63 western analysis indicated that ectopic ALK5 resulted in phosphorylation of Δ Np63 α . Remarkably, a threonine to aspartate mutation T202D (T198D in rat ALK5 NP_036907.2) that constitutively activates TGF β signaling [34] caused an increase in SMAD2 phosphorylation but was unable to phosphorylate Δ Np63 α . Additionally a lysine to arginine mutation K232R (K226R in rat ALK5 NP_036907.2) in rat ALK5 that fails to mediate canonical TGF β signaling [35] and fails to phosphorylate SMAD2 but is able to efficiently phosphorylate Δ Np63 α (Figure S2). These observations suggest that the intramolecular determinants of SMAD2 phosphorylation, and by extension canonical TGF β -signaling, are distinct from those required for Δ Np63 α phosphorylation. Together these studies demonstrate that ALK5 is necessary and sufficient to phosphorylate Δ Np63 α and suggest that the molecular mechanisms by which ALK5 phosphorylates Δ Np63 α may be distinct from those governing phosphorylation of SMAD2.

TGF β Stimulates ALK5-mediated Phosphorylation of Δ Np63 α via TGF β R2

The finding that ALK5 is necessary and sufficient for Δ Np63 α phosphorylation suggested that TGF β -signaling governs Δ Np63 α phosphorylation. We therefore sought to determine if TGF β stimulation was sufficient to enhance Δ Np63 α phosphorylation. For these studies, an hTERT-immortalized mammary epithelial cell (IMEC) line was used due to its robust expression of Δ Np63 α [32] and the fact that it is cultured in a chemically-defined media, which enables experimentation under TGF β -depleted conditions. Results indicate that TGF β stimulation increases phospho- Δ Np63 α levels within one hour, indicating signaling kinetics similar to SMAD2 phosphorylation. This phosphorylation was inhibited by A83-01, a selective ALK5 kinase inhibitor [36] (Figure 2B), which supports the assertion that TGF β mediated Δ Np63 α phosphorylation requires ALK5 activity. This observation coupled to the fact that ALK5 possesses no inherent TGF β -binding capacity suggested the involvement of TGF β R2. SiRNAs that produce a substantial reduction in TGF β R2 expression (Figure S3) directed against TGF β R2 were co-transfected into IMECs $\pm$ pcDNA- Δ Np63 α . Phospho-p63 western analysis indicated that TGF β R2 is necessary for TGF β -mediated phosphorylation of Δ Np63 α and SMAD2 (Figure 2C). Under TGF β -

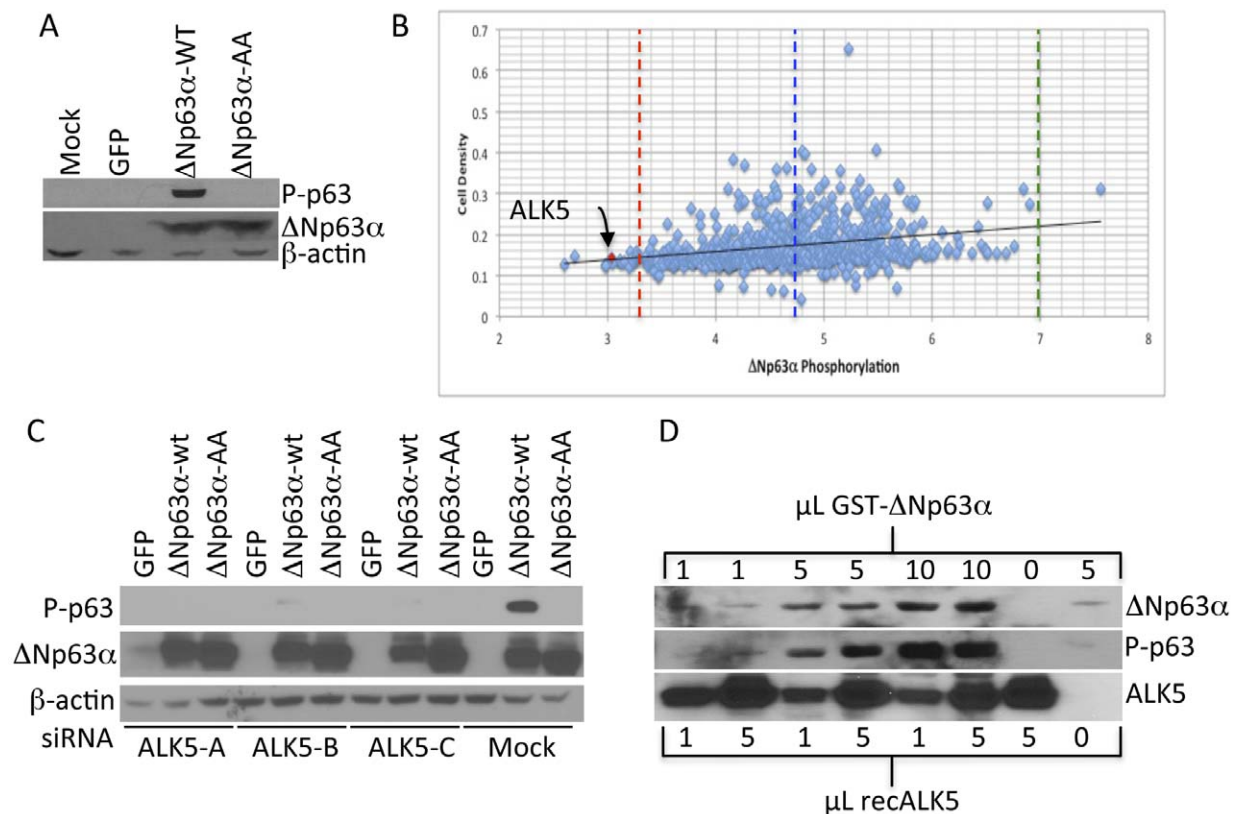


Figure 1. An siRNA-based screen of the human kinome identifies ALK5 as a putative Δ Np63 α kinase. **A.** Δ Np63 α -WT and phospho-ablative p63 mutant (Δ Np63 α -AA) were transiently transfected into H1299 cells. Cell lysates were harvested after 24 hrs and the levels of phospho-P63, total p63 and β -actin were analyzed. **B.** Graphical representation of results of the phospho-P63 expression as indicated by normalized fluorescent values from the kinome screen. Each dot represents relative p-p63 abundance following treatment with siRNA ($n = 3$) directed against a single kinase. The green dashed line represents mean positive control value and the red dashed line represents the mean negative control. The blue dashed line represents the mean phospho-p63 score in the screen. All the hits below the red dotted line were considered as possible kinases responsible for phosphorylating Δ Np63 α . **C.** Three different siRNAs targeted against ALK5 were transfected into H1299 cells, 48 hrs later cells were transfected with either GFP, Δ Np63 α -WT or Δ Np63 α -AA expression vectors, whole cell lysates harvested after 24 hrs were analyzed for phospho-P63, total p63, and β -actin. **D.** Recombinant ALK5 phosphorylates Δ Np63 α in vitro. Purified GST tagged Δ Np63 α protein was incubated with purified Flag-ALK5 kinase for 30 min at 30°C and reactions were analyzed for phospho-P63, totalP63, and Flag-ALK5. doi:10.1371/journal.pone.0050066.g001

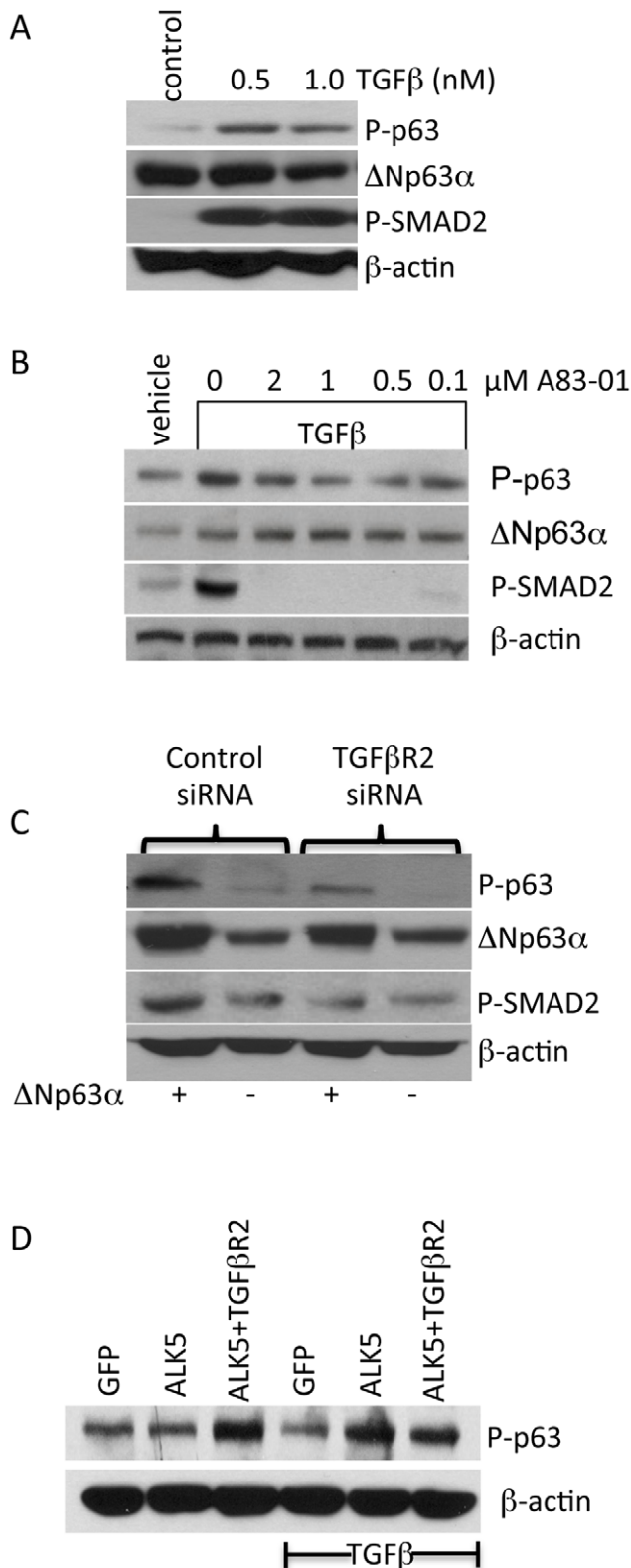


Figure 2. TGFβ stimulates of ALK5-mediated phosphorylation of Δ Np63 α via TGFβR2. **A.** IMEC cells were treated with the indicated concentrations of TGFβ1 ligand for 1 hr. Whole cell lysates were harvested and analyzed by immunoblotting for levels of phospho-p63, total p63, phospho-SMAD2 and β-actin. **B.** ALK5-mediated phosphorylation of Δ Np63 α is inhibited by A83-01. IMEC cells were treated with A83-01 at the indicated concentrations 1 hr prior to TGFβ1 treatment.

Whole cell extracts were analyzed after 1 hr for phospho-p63, total-p63, phospho-SMAD2 and β-actin via immunoblotting. **C.** siRNA targeted against TGFβR2 was transfected into IMEC cells, 48 hrs later cells were transfected with Δ Np63 α expression vector or GPP control vector. Whole cell lysates were harvested 24 hrs later and analyzed for phospho-P63, total-P63, phospho-SMAD2 and β-actin. **D.** Ectopic expression of ALK5 and TGFβR2 is sufficient to phosphorylate Δ Np63 α in a manner that is independent of TGFβ. doi:10.1371/journal.pone.0050066.g002

depleted culture conditions ectopic TGFβR2 promoted ALK5-mediated phosphorylation of Δ Np63 α in a manner that was independent of TGFβ (Figure 2D, compare lanes 2 and 3). This result is consistent with studies indicating that TGFβ stimulates the physical association of TGFβR2 and ALK5 [37]. Together, these results indicate that TGFβ initiates ALK5-mediated Δ Np63 α phosphorylation via the canonical TGFβR2/ALK5 receptor complex.

ALK5 Mediates Phosphorylation of Δ Np63 α in Response to Ultraviolet Irradiation

Previous studies have shown that Δ Np63 α is phosphorylated at S66/68 in response to ultraviolet (UV) irradiation [29]. Other studies have demonstrated increased TGFβ signaling in response to UV irradiation [38–40]. Additionally, recent studies have implicated TGFβ signaling in increased metastasis following ionizing radiation [41] and others have shown that ionizing radiation results in enhanced TGFβ signaling from the tumor microenvironment that results in pro-carcinogenic effects [42]. Together these studies indicate that ALK5 signaling mediates Δ Np63 α phosphorylation in response to UV irradiation. To determine if ALK5 mediates UV-induced phosphorylation of endogenous Δ Np63 α , IMECs were incubated in the presence or absence of A83-01 for 12 hrs and then subjected to UV irradiation. Western analysis indicated that UV irradiation stimulated phosphorylation of Δ Np63 α was sensitive to A83-01, indicating that ALK5 was able to phosphorylate Δ Np63 α in response to UV irradiation (Figure 3A). Interestingly, UV irradiation also increased phosphorylation of SMAD2 in a manner that was sensitive to A83-01 suggesting that the mechanism(s) by which UV irradiation induces TGFβ-signaling most likely act upstream of ALK5. Similarly H1299 cells were transfected with GFP, Δ Np63 α -WT or the phospho-ablative mutant, Δ Np63 α -AA, and cells were either mock exposed or exposed to UV irradiation followed by treatment with A83-01 or vehicle. Western blot analysis indicated that phosphorylation of Δ Np63 α at S66/68 increased in response to UV irradiation and that inhibition of ALK5 with A83-01 was sufficient to ablate Δ Np63 α phosphorylation (Figure 3B). Together these studies indicate that ALK5 mediates UV-induced activation of TGFβ-signaling which leads to increased phosphorylation of SMAD2 and Δ Np63 α . These studies implicate ALK5 in a previously unknown role in the cellular stress response and suggest that disruption of Δ Np63 α phosphorylation may sensitize cells to diverse types of cellular stress. Coupled to studies indicating that Δ Np63 α is a potent blockade to apoptosis in experimental models of HNSCC [16] and triple negative breast cancer [15] these studies identify a potential strategy to subvert Δ Np63 α mediated drug resistance by inhibiting TGFβ signaling.

Nuclear Accumulation of the Intracellular Kinase Domain of ALK5 (ALK5^{IKD}) in Response to TGFβ

The identification of ALK5 as a Δ Np63 α kinase and the demonstration that this event is initiated by TGFβ raised significant questions regarding the mechanisms by which a

membrane bound kinase phosphorylates Δ Np63 α , which is located in the nucleus. ALK5 activation is propagated through multiple transduction pathways, several of which rely upon diverse kinase activities [43–45]. To determine if any of these known pathways mediate Δ Np63 α phosphorylation, phospho- Δ Np63 α immunofluorescence data from the kinome-wide siRNA screen was re-evaluated. Results of this evaluation indicated that no other kinase known to be downstream of ALK5 was implicated in Δ Np63 α phosphorylation (Figure S4). Additionally, western analysis of IMECs transfected with ALK5 siRNA identified a 34-kDa fragment that was sensitive to ALK5-directed siRNA and detectable with an antisera directed against the C-terminus of ALK5 (Figure 4A). The 34-kDa size coupled to selective detection with a C-terminally directed antibody suggested that this band is the product of proteolytic cleavage of ALK5 at or near the intracellular juxtamembrane region. To test this hypothesis, a plasmid encoding C-terminally-flag-tagged versions of wild-type ALK5 was transfected into H1299 cells and flag-tagged proteins were detected by western blot. Results indicated that ectopic expression of ALK5 results in a 56 kDa full-length receptor and a 34 kDa C-terminal fragment (Figure 4B) suggesting that exogenous ALK5 was processed in a manner that is similar or identical to endogenous ALK5. Since these experiments were done by transfecting cDNAs of wild-type and mutant ALK5, these results also demonstrate that the 34-kDa fragment is unlikely to be the result of alternative mRNA splicing. These observations support a model in which TGF β stimulates proteolytic cleavage of ALK5 and that the 34 kDa ALK5 intracellular kinase domain (ALK5^{IKD}) would preferentially localize in the nucleus. Western analysis of nuclear and cytoplasmic extracts from IMECs indicated that the 34-kDa ALK5 C-terminal fragment was selectively localized to the nucleus (Figure 4C). To determine if ALK5 is able to translocate to the nucleus, an ALK5-GFP fusion expression vector [46] was transfected into IMECs under TGF β -depleted conditions and cells were then stimulated with vehicle or TGF β . Fluorescence microscopy indicated that TGF β stimulated the redistribution of ALK5-GFP the nucleus, consistent with the nuclear localization of the ALK5^{IKD} (Figure 4D). Similarly, stimulation of H1299 cells with TGF β resulted in redistribution of ALK5 from the cytoplasm to the nucleus (Figure 4E). Transfection of ALK5-directed siRNA confirms the specificity of the immuno-

fluorescent analysis (Figure S5). Together these observations support a mechanistic model in which TGF β stimulation initiates the nuclear translocation of ALK5, thereby enabling phosphorylation of Δ Np63 α .

TGF β is Anti-proliferative and Suppresses ALDH1 Activity and Δ Np63 α Protein Levels in a Mammary Stem Cell Model

The hTERT immortalized mammary epithelial cells (IMECs) were derived via retroviral transduction of the catalytic subunit of human telomerase (hTERT) into primary human mammary epithelia and clones were selected for their ability to bypass replicative senescence [32]. Subsequent analysis of multiple clonal IMEC lines indicated a basal/myoepithelial cytokeratin profile and robust expression of Δ Np63 α . Other studies indicated that IMECs possess developmental potency based upon their ability to produce acinar structures with biochemically distinct basal and luminal layers [47]. Based upon these similarities to mammary stem cells, we sought to understand the biological effects of TGF β on IMECs and to determine the degree to which phosphorylation of Δ Np63 α contributes to these effects. TGF β caused a significant decrease in cell number over 72 hours, and this effect was reversed by co-treatment with A83-01 (Figure 5A). Cell cycle distribution analysis indicated a decrease in the population of cells in S-phase in response to TGF β and a corresponding increase of cells in S-phase in response to A8301 (Figure 5B). To address the effects of TGF β on stem cell activity, IMEC sub-populations with features of stem cells were enriched on the basis of high aldehyde dehydrogenase 1 (ALDH1) activity [48]. Analysis of Δ Np63 α mRNA levels in ALDH1^{high} and ALDH1^{low} fractions indicated that Δ Np63 α mRNA levels were significantly enriched in the ALDH1^{high} self-renewing population (Figure 5C). This enrichment for Δ Np63 α expression is consistent with the assertion that ALDH1^{high} fractions of IMECs are enriched for self-renewing capacity and also indicates that TGF β may influence this fraction via Δ Np63 α phosphorylation. To test this, IMECs were treated with vehicle, TGF β or A83-01 for 24 and 48 hours, and the ALDH1^{high} fraction was measured. Results indicated that TGF β treatment significantly reduced ALDH1 activity in IMECs resulting in a smaller ALDH1^{high} cellular fraction (Figure 5D and Figure S6) The reduction in ALDH1^{high} cells in response to

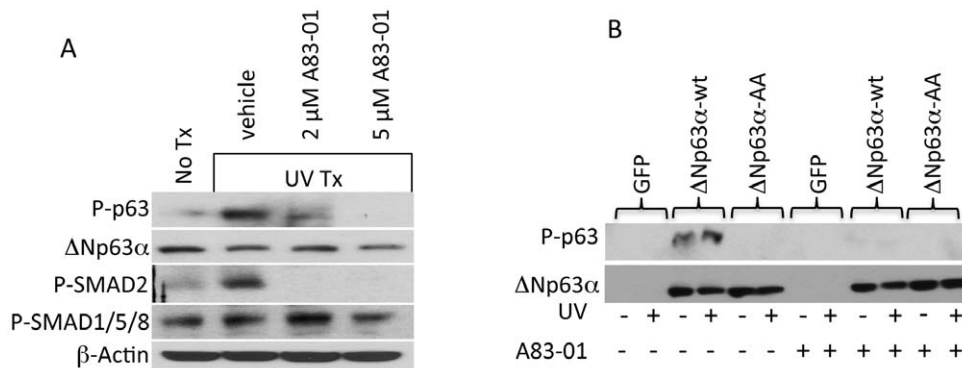


Figure 3. ALK5 mediates phosphorylation of Δ Np63 α in response to ultraviolet irradiation. **A.** IMEC cells were plated and treated with the indicated amounts of A83-01 or vehicle for 12 hrs after which cells were exposed to 50 J/m² UV radiation. Whole cell lysates were collected after 1 hr of UV treatment and analyzed by immunoblotting for phospho-P63, total-P63, phospho-SMAD2 and β -actin levels. Phospho-SMAD1/5/8 was used as a control to show that there were no off-target effects for A83-01 at the concentrations used. **B.** H1299 cells were transfected with GFP, Δ Np63 α -WT or Δ Np63 α -AA expression vectors and cells were treated with 2 μ M A83-01 or vehicle control. Twenty-four hours later cells were exposed to 50 J/m² UV radiation and whole cell extracts were collected after 1 hr to analyze the levels of phospho-P63, total-P63 and β -actin by immunoblotting.

doi:10.1371/journal.pone.0050066.g003

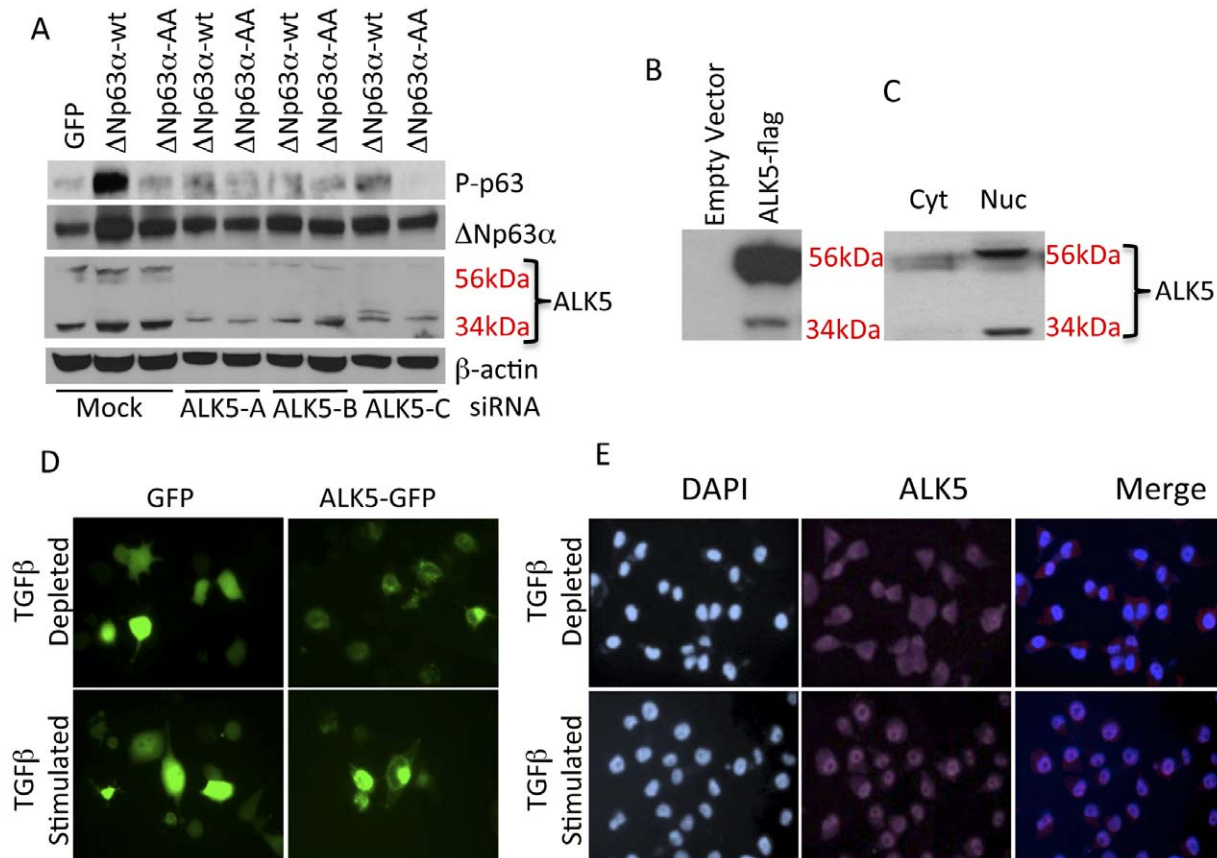


Figure 4. Nuclear accumulation of the intracellular kinase domain of ALK5 (ALK5^{IKD}) in response to TGF β . **A.** Three different siRNAs targeted against ALK5 were transfected into IMEC cells, 48 hrs later cells were transfected with GFP, Δ Np63 α -WT and Δ Np63 α -AA expression vectors, whole cell lysates were harvested after 24 hrs and analyzed for phospho-p63, total p63, TGF β R1 and β -actin. **B.** H1299 cells were transfected with an ALK5-WT-Flag expression vector, whole cell lysates were harvested and analyzed for full length and cleaved fragments with anti-Flag antibody. **C.** Analysis of ALK5 distribution in IMECs indicates that the 34 kDa C-terminal ALK5 fragment is present only in the nucleus. **D.** H1299 cells were transfected with ALK5-GFP expression vector, 6 hrs after transfection cells were treated with vehicle control or TGF β 1 for 8 hrs and imaged for subcellular distribution of GFP. **E.** H1299 cells were serum starved for 12 hr and then induced with vehicle control or TGF β 1 for 1 hour. Cells were stained with anti- TGF β R1 (V-22) antibody. Depletion of TGF β R1 in H1299 cells by siRNA is shown as a control for the specificity of the antibody. doi:10.1371/journal.pone.0050066.g004

TGF β , coupled to the fact that Δ Np63 α expression is increased in this fraction suggested that TGF β might be opposing the activity of Δ Np63 α . Together these data indicate that TGF β signaling in IMECs is anti-proliferative and targets self-renewing populations that are enriched for Δ Np63 α expression.

The previous data suggests that TGF β opposes the activity or expression of Δ Np63 α . This coupled to previous studies indicating that phosphorylation of Δ Np63 α at S66/68 leads to its destabilization suggested that TGF β might oppose Δ Np63 α by causing its degradation. To test this, IMECs were treated with TGF β or A83-01 in the absence or presence of cycloheximide. Under these conditions treatment of IMECs with TGF β for 4 hours in the absence of *de novo* protein synthesis selectively repressed Δ Np63 α protein levels indicating that TGF β may destabilize Δ Np63 α (Figure 6A). To determine if the phosphorylated form of Δ Np63 α was preferentially destabilized, cells were pre-treated with vehicle, TGF β or A83-01 for 1 hour followed by treatment with vehicle or cycloheximide for 4 hours and phospho-p63 levels were evaluated by western blot. Consistent with data in Figure 2, TGF β stimulation leads to increased phosphorylation of Δ Np63 α , however, treatment with cycloheximide resulted in the destabilization of phospho-p63 signal in the TGF β -treated sample, indicating that TGF β -mediated phosphorylation of Δ Np63 α

destabilizes Δ Np63 α (Figure 6B). To determine if TGF β -stimulated degradation of Δ Np63 α was mediated by the 26S proteasome, H1299 cells were transfected with GFP, Δ Np63 α -WT and Δ Np63 α AA under conditions that actively promoted TGF β signaling. Cells were then treated for 2 hours with vehicle or 1 μ M MG-132. Western analysis showed that phosphorylated Δ Np63 α was stabilized by MG132 indicating that phospho- Δ Np63 α is degraded by the 26S proteasome. Western analysis for total Δ Np63 α indicated that MG132 caused an increase in Δ Np63 α -WT but not in Δ Np63 α -AA, indicating that targeting of Δ Np63 α to the 26S proteasome requires phosphorylation of serines 66 and 68. These findings support a model in which TGF β -mediated phosphorylation of Δ Np63 α leads to its degradation by the 26S proteasome.

The Anti-clonogenic Effects of TGF β Require Phosphorylation of Δ Np63 α

TGF β signaling has been shown to be a tumor suppressive during early stages of breast cancer initiation and to promote breast cancer progression and metastasis at later stages [18]. The specific targets and signaling pathways governing these divergent effects are incompletely understood. The previous results indicate that TGF β signaling is growth inhibitory and destabilizes Δ Np63 α

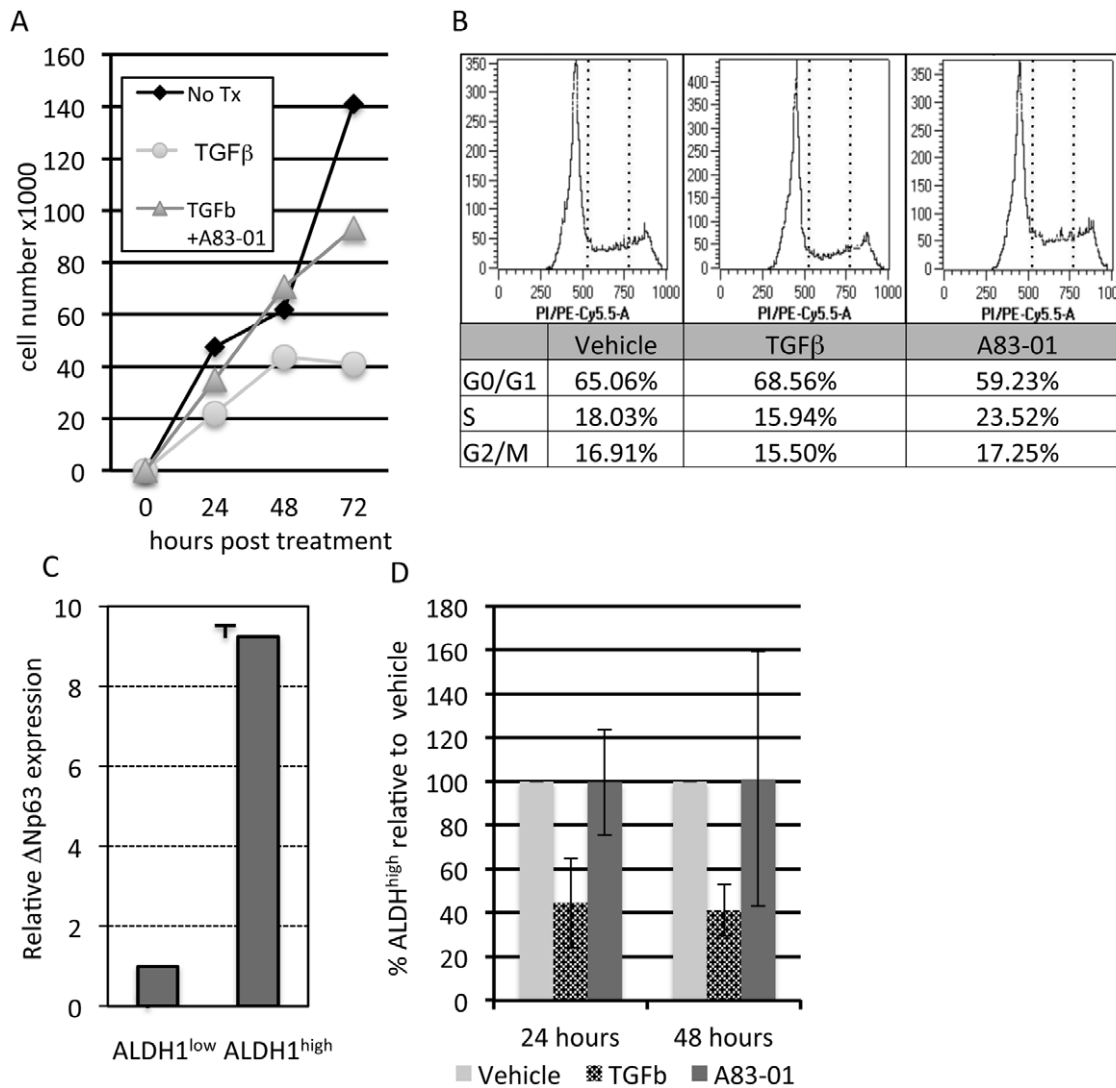


Figure 5. TGF β is anti-proliferative and suppresses ALDH1 and Δ Np63 α protein levels activity in a mammary stem cell model. **A.** Cell counts plotted for IMEC cells treated with control, TGF β 1 and A83-01 for the indicated time points. Data are representative of multiple **B.** Cell cycle analysis was performed by PI staining on IMEC cells after treatment with control, TGF β 1 or A83-01 for the indicated time points. Dashed lines flank the S-phase region. **C.** IMEC cells were sorted based on the ALDH staining, Δ Np63 α mRNA expression levels were analyzed using quantitative PCR. **D.** IMEC cells expressing ALDH high and low were analyzed after treating IMEC cells with vehicle control, TGF β 1 and A83-01 at the indicated time points. **E.** IMEC cells were treated with control or cycloheximide (CHX) for 2 hrs and then treated with TGF β 1 or A83-01 for 2 hrs. Whole cell lysates were analyzed for Δ Np63 α and β -actin. **F.** IMEC cells were treated with control, TGF β 1 and A83-01 for 1 hr, after which cells were treated with control and CHX for 4 hrs. Whole cell lysates were analyzed for Phospho-p63 and β -actin.
doi:10.1371/journal.pone.0050066.g005

via phosphorylation. To determine if phosphorylation of Δ Np63 α is required for the anti-proliferative effects of TGF β , IMECs were transfected with GFP, Δ Np63 α -WT or Δ Np63 α -AA and selected in G418 while simultaneously being treated with either TGF β or A83-01. Colonies were allowed to grow for 15 days and then fixed and stained with crystal violet. Consistent with the observed effects on proliferation and ALDH1 activity, TGF β treatment was anti-clonogenic, while A83-01 promoted colony formation (Figure 7A and Figure S7A). Additionally, the anti-clonogenic effect of TGF β was rescued by Δ Np63 α -AA but not Δ Np63 α -WT (Figure 7A). To determine if these differences were statistically significant, a two-tailed T-Test of the effects of TGF β on colony formation in each transfection group revealed that TGF β caused a statistically significant reduction in colony formation in the GFP transfectant

($P = 0.00167$) and the Δ Np63 α -WT transfectant ($P = 0.000433$), but not in the Δ Np63 α -AA transfectant ($P = 0.4676$). This statistical analysis supports the assertion that Δ Np63 α -AA was able to rescue the anti-clonogenic effects of TGF β . The coupled to the finding that Δ Np63 α -WT was unable to rescue these effects supports the conclusion that the anti-clonogenic effects of TGF β require phosphorylation of Δ Np63 α at serine 66 and 68. This result indicates that the anti-clonogenic effects of TGF β require Δ Np63 α phosphorylation. This observation coupled to data indicating that TGF β stimulates nuclear translocation of ALK5 suggests that the intracellular kinase domain of ALK5 (ALK5^{IKD}) mediates TGF β -stimulated phosphorylation of Δ Np63 α and the anti-clonogenic effects of TGF β . To address this, an expression vector was developed to produce ALK5^{IKD} and transfected into

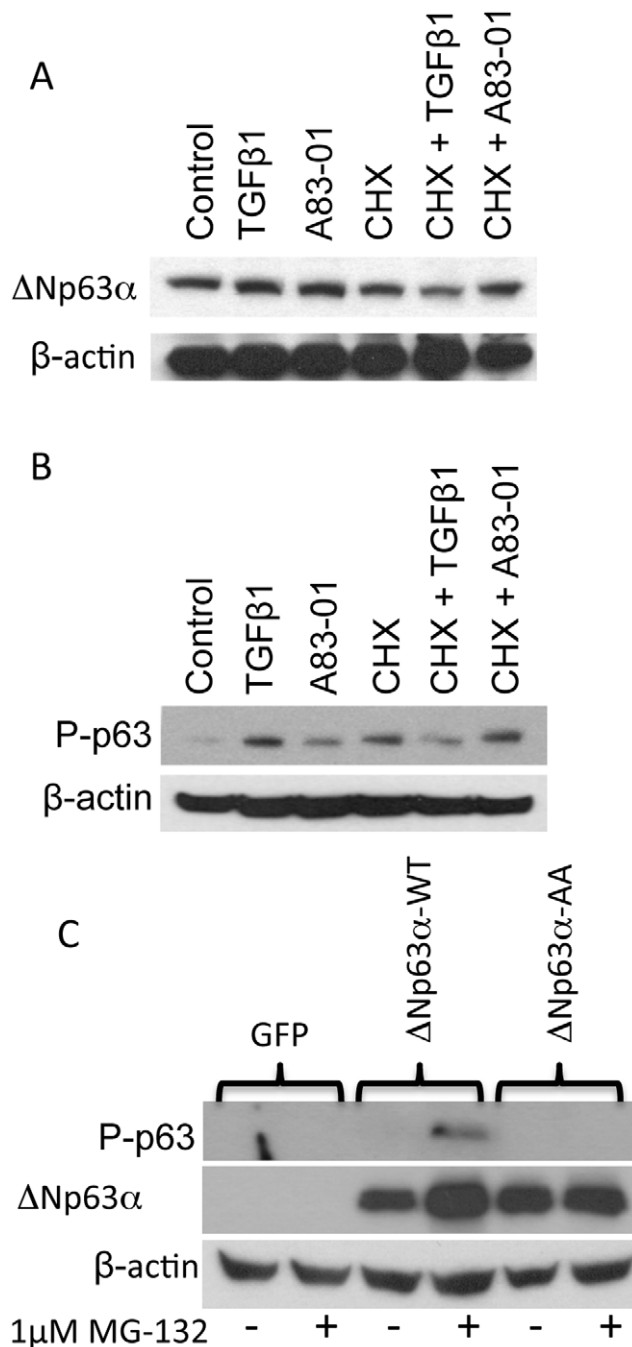


Figure 6. TGF β treatment destabilizes Δ Np63 α in a manner that is dependent upon the kinase activity of ALK5. **A.** TGF β increases the rate of Δ Np63 α turnover. IMECs were treated with vehicle or cycloheximide followed by treatment with vehicle, TGF β or A8301. **B.** TGF β -stimulation selectively increases the rate of turnover of phospho- Δ Np63 α . IMECs were treated with vehicle, TGF β or A83-01 followed by vehicle or cycloheximide. **C.** Phospho-p63 is stabilized by the 26S proteasome inhibitor MG132. H1299 cells were transfected with GFP, Δ Np63 α WT and Δ Np63 α AA and treated for 2 hours with 1 μ M MG-132. doi:10.1371/journal.pone.0050066.g006

IMECs. Consistent with the proposed model, ectopic ALK5^{IKD} accumulates in the nucleus (Figure 6B) and phosphorylates Δ Np63 α in a manner that is independent of TGF β but sensitive

to A83-01 (Figure 6C). The observation that ectopic ALK5^{IKD} is sufficient to phosphorylate Δ Np63 α under TGF β -depleted conditions is consistent with a model in which TGF β signaling is bypassed by directly targeting ALK5^{IKD} to the nucleus. This observation predicts that ALK5^{IKD} is sufficient to recapitulate the anti-clonogenic effects of TGF β . IMECs were transfected with expression plasmids encoding ALK5^{IKD} and either Δ Np63 α -WT or Δ Np63 α -AA. G418 resistant colonies were selected and quantified. Results indicated that ALK5^{IKD} was potentially anti-clonogenic (Figure 6D) and that this effect was partially rescued by Δ Np63 α -WT and completely rescued by Δ Np63 α -AA (Figure 6E). These results confirm that the anti-clonogenic effects of TGF β in IMECs are mediated by ALK5^{IKD} and require phosphorylation of Δ Np63 α . These studies identify Δ Np63 α as a novel target of TGF β signaling and indicate that the ability of Δ Np63 α to promote colony formation is potentially inhibited by TGF β .

Discussion

We report the identification of the Type 1 TGF β Receptor, ALK5, as a kinase that mediates phosphorylation of Δ Np63 α at S66/68. Our studies indicate that TGF β stimulation and UV irradiation also phosphorylate Δ Np63 α at S66/68 and this effect is sensitive to pharmacologic inhibition of ALK5 with A83-01. We present data indicating that TGF β is able to stimulate the nuclear translocation of ALK5 and that a 34 kDa C-terminal truncation of ALK5 preferentially translocates to the nucleus. Our studies indicate that the anti-clonogenic effects of TGF β are mediated by Δ Np63 α phosphorylation. Coupled to the established role of Δ Np63 α in the long-term preservation of proliferative capacity in adult stem cells, these studies suggest that TGF β /ALK5/ Δ Np63 α signaling may contribute to the proliferative capacity of adult stem cells and tumor stem cells. Together these studies describe a previously unrecognized TGF β signaling pathway that directly impacts the proliferative capacity and clonogenicity of Δ Np63 α -positive cells. Additional studies will be necessary to determine the degree to which this pathway accounts for the effects of TGF β on the activity of adult stem cells. Previous studies have shown that TGF β promotes oncogene-induced senescence (OIS) in a manner that is independent of p53 [49]. Separately Δ Np63 α has been shown to be a potent suppressor of OIS [13]. Here we present data indicating that TGF β activation destabilizes Δ Np63 α suggesting a potential mechanism by which TGF β promotes OIS. This report establishes a novel signaling relationship between TGF β and TP63 and demonstrates that this relationship underlies aspects of adult stem cell biology that are governed by Δ Np63 α . Finally it will be important to elucidate the role of this signaling pathway in Epithelial to Mesenchymal Transition. Recent studies indicate that Δ Np63 α opposes EMT [50–51] suggesting that TGF β -mediated destabilization of Δ Np63 α may be an important step in EMT, a process that is critical for cancer progression and metastasis. Additional studies will be necessary to systematically evaluate the cellular consequences of TGF β -mediated phosphorylation of Δ Np63 α .

Previous studies have shown that Δ Np63 α is phosphorylated at S66/68 in response to UV irradiation [29]. The observation that UV-initiated phosphorylation of Δ Np63 α was sensitive to A83-01 implicates ALK5 in the cellular response to stress, however, additional studies will be necessary to determine if ALK5-mediated Δ Np63 α phosphorylation contributes to the role of TGF β signaling in promoting metastasis following ionizing radiation [41]. Similarly, it will be of significant interest to determine if the potent TGF β response of the tumor microenvironment to radiation [42] contributes to Δ Np63 α phosphoryla-

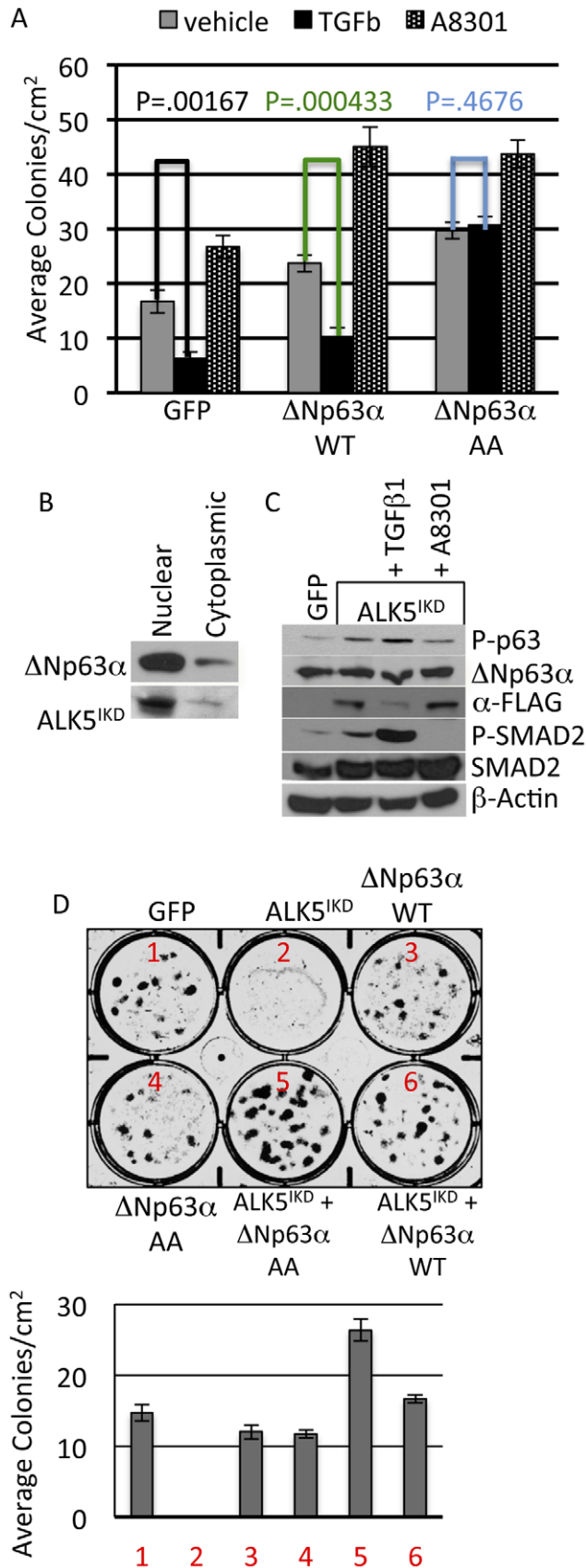


Figure 7. The anti-clonogenic effects of TGF β require phosphorylation of Δ Np63 α . **A.** IMEC cells were transiently transfected with GFP, Δ Np63 α -WT or Δ Np63 α -AA at equimolar ratios. 48 hrs after transfection cells were subjected to G418 selection with control, TGF β 1 or A8301. Colonies formed were then stained with crystal violet. To quantify colonies 3 randomly 1 cm² spaces per well were counted and these data were used to calculate the average number of colonies per cm². Error bars represent the standard deviation. A two-tailed T-Test was used to determine the significance of the effects of TGF β on colony formation in each transfection group. **B.** ALK5^{IKD}-flag expression vector was transiently transfected into IMEC cells, 24 hrs later cells were harvested for nuclear and cytoplasmic extracts. Subcellular localization of P63 and ALK5^{IKD} proteins were analyzed by immunoblotting with Anti-P63 and Anti-Flag antibodies. **C.** IMEC cells were transfected with GFP and ALK5^{IKD}, 24 hrs later cells were treated with TGF β 1 and A8301 for 1 hr and whole cell lysates were analyzed for phospho-P63, total P63, phospho-SMAD2, total SMAD2 and β -actin via immunoblotting. **D.** IMEC cells were transfected with GFP or ALK5^{IKD} at equimolar ratios. 48 hrs after transfection cells were subjected to G418 selection until visible colonies formed which were then stained with crystal violet. **E.** IMEC cells were either transfected with GFP, ALK5^{IKD}, Δ Np63 α -WT and Δ Np63 α -AA or ALK5^{IKD}+ Δ Np63 α -AA and ALK5^{IKD}+ Δ Np63 α -WT at equimolar ratios. 48 hrs after transfection cells were subjected to G418 selection. Formed colonies were then stained with crystal violet. doi:10.1371/journal.pone.0050066.g007

tion. Coupled to the finding that TGF β -mediated phosphorylation of Δ Np63 α is anti-proliferative and anti-clonogenic, the ALK5-mediated response to cellular stress may act as a protective mechanism that limits proliferation under conditions of cellular or genotoxic stress. Additional studies will be necessary to determine if the mechanisms underlying activation of ALK5 in response to cellular stress are TGF β -dependent or TGF β -independent. Furthermore, it will be potentially clinically relevant to determine the biological consequences of ALK5-mediated phosphorylation of Δ Np63 α in enriched tumor stem cell fractions. Finally, Δ Np63 α has been shown to act as a survival factor that mediates therapeutic resistance and opposes apoptosis in breast cancers with a basal phenotype [15] and also in squamous cell carcinomas of the head and neck [16]. It will be of interest to determine if disruption of the TGF β /ALK5/ Δ Np63 α signaling pathway subverts these activities thereby overcoming therapeutic resistance.

Finally, recent studies have indicated unacceptable levels of cardiac and inflammatory toxicity associated with selective ALK5 kinase inhibitors and it is likely that these adverse effects will limit their development and clinical utility [52]. Given the remarkably pleiotropic actions of TGF β , it is not surprising that drugs that disrupt all ALK5 signaling would have a wide range of effects. This highlights the need to identify specific signaling pathways downstream of TGF β that account for specific activities of TGF β . Doing so will make it possible to target specific actions of TGF β while avoiding adverse side effects. Data presented here support a model in which unknown proteolytic activity mediates the translocation of ALK5^{IKD}. This implies that inhibition of this protease may result in disruption of Δ Np63 α phosphorylation. Further studies will be necessary to identify this protease, however a recent study has shown that the TNF- α Converting Enzyme (TACE) is able to mediate proteolysis of ALK5 and that TACE activity is required for accumulation of ALK5 in the nucleus [53]. The specific relevance of this finding to the generation of ALK5^{IKD} and phosphorylation of Δ Np63 α is unknown because in that study TACE was shown to target the ALK5 ectodomain, which is predicted to produce a fragment greater than 34 kDa. This also raises questions regarding the mechanism(s) by which an ALK5 fragment that retains the transmembrane domain might translocate to the nucleus. Presently the protease(s) that account

for generation of ALK5^{IKD} remain unknown and their identification represents an important step in testing our model for ALK5-mediated Δ Np63 α phosphorylation and also in identifying pharmacologically accessible pathway components.

Supporting Information

Figure S1 Schematic representation of the siRNA-based screen of the human kinome. The table at the bottom lists the top 14 hits in the screen showing the data produced from fluorescence plate readings of the IF and subsequent Abs₆₀₀ readings for crystal violet staining. Primary hits progressed to the secondary screen and kinases for which all three siRNAs repressed phosphorylation of DNp63 α were selected. (PDF)

Figure S2 The molecular determinants of DNp63 α phosphorylation by ALK5 are distinct from those necessary for SMAD2/3 phosphorylation. H1299 cells were co-transfected with wild-type ALK5, the T202D mutant which had previously been shown to constitutively activate TGF β signaling, the K232R mutant which had been previously shown to inhibit TGF β signaling and an ALK5-GFP fusion. At 24 hours post transfection protein was harvested and analyzed by western blot. Comparison of the P-p63 and P-SMAD2/3 signals indicated that T202D was unable to phosphorylate DNp63 α but was able to phosphorylate SMAD2/3 (Lane 3). Remarkably the K232R mutant was able to phosphorylate DNp63 α but not SMAD2/3 (Lane 4). These results suggest that the molecular mechanisms by which ALK5 phosphorylates DNp63 α are distinct from those that phosphorylate SMAD2/3. (PDF)

Figure S3 Effects of three TGF β R2-directed siRNAs on expression of TGF β R2 and SMAD2 phosphorylation. H1299 cells were transfected with the indicated siRNAs and TGF β R2 and phospho-SMAD2 were analyzed to confirm the efficacy of the siRNA. SiRNA-C was used in the experiment shown in Figure 2C. (PDF)

Figure S4 Schematic representation of signal transduction pathways known to be downstream of the TGF β receptor complex. Kinases associated with these pathways are shown in

pink and the phospho-p63 vs total p63 IF score is shown as is the relationship of that score to the mean. (PDF)

Figure S5 Transfection of H1299 cells with ALK5-directed siRNA ablates immunofluorescent detection of ALK5. This data confirms the specificity of ALK5 detection presented in Figure 4E. This data confirms the selectivity of the ALK5 antibody. (PDF)

Figure S6 Representative Aldefluor data from which Figure 5D was derived. Negative controls using the ALDH1 inhibitor DEAB are used to establish the gate separating ALDH^{Low} from ALDH^{High} fractions. (PDF)

Figure S7 The anti-clonogenic effects of TGF β are phenocopied by ectopic ALK5IKD. **A.** The anticlonogenic effects of TGF β on IMECs are partially rescued by the phospho-ablative DNp63 α -AA mutant. Colony forming assay shown is representative of multiple experiments and corresponds to the graphical data displayed in Figure 7A. **B.** Ectopic expression of ALK5^{IKD} is anti-clonogenic in IMEC cells. IMECs were transfected with pcDNA3.1-GFP and pcDNA3.1-ALK5^{IKD} and selected in 200 μ g/ml G418 for 12 days. Colonies were fixed in alcohol and stained with crystal violet. Graph at right represents a quantification of the colony formation in which colonies from three random 1 cm $\times$ 1 cm squares were analyzed using ImageJ software. Bars represent the average of three counts and error bars represent the standard error of the mean. (PDF)

Acknowledgments

The authors wish to thank Dr. Lalage Wakefield for valuable advice and expertise in TGF β signaling.

Author Contributions

Conceived and designed the experiments: JDR PC. Performed the experiments: PC SLD JYL AJD. Analyzed the data: ALB JAH. Wrote the paper: JDR PC.

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