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TITLE: Twist Maintains Stemness in Latent Breast Cancer Metastases Through a Novel Non DNA-Binding Function Targetable With Small Molecules

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14. ABSTRACT

The Twist induced EMT program in a primary tumor is required for proliferation, migration, invasion and colonization of distant organs. Once cells are disseminated, a genetically and biochemically separate domain of Twist: the WR domain functions to maintain the metastatic tumor cells in a quiescent (possibly pluripotent) state by rendering the cells resistant to differentiation inducing signals, similar to what happens during development. The hypothesis and indeed preliminary data provided to support the original proposal were that the COOH-terminal Twist WR domain is required for binding directly to lineage specific transcription factors like Runx2 and inhibiting their DNA binding function. Thus, the goals of this grant were to define how Twist and the WR domain functions to interact with other cellular factors and maintain the undifferentiated state in these latent disseminated cells. Progress in this second year of support includes: 1. Further definition of the spectrum of proteins bound by to endogenous, cell derived Twist in a breast cancer cell line undergoing EMT. 2. Reconstitution of this complex with purified recombinant Twist-E47-Snail-Sox9 proteins. 3. The surprise discovery that this complex of proteins does NOT function to inhibit Runx1 DNA binding as originally proposed. Rather, the protein complex transiently binds to Runx2 and leads to its proteasome mediated degradation. The multiprotein complex does not require any intrinsic DNA binding capability to target and degrade Runx2, so it is the first NON DNA binding dependent function described for these transcription factors. To investigate the mechanism of proteasome mediated degradation of Runx2 by Twist-WR (and its binding proteins). Preliminary data suggest that the Snail component of the Twist complex functions as an E3 ligase for Runx2 and recruits it to the proteasome. This E3 ligase activity resides solely in 2-4 zinc fingers of Snail and is highly specific for Snail family members. Thus, we sought to determine the exact mechanism of Snail E3 ligase activity towards Runx1.

15. SUBJECT TERMS
Breast Cancer, Metastasis, EMT, differentiation, stem cell, Twist, RUNX, Gene Regulation

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1. **INTRODUCTION:** We hypothesized that the Twist WR domain is required for establishment and maintenance of the stemness phenotype that is induced in breast cancer cells as a result of the EMT program and that the WR domain functions as a protein-protein interface that binds to and inhibits lineage specific differentiation transcription factors, thereby maintaining the stemness state. The overall objective was to define the mechanism by which the WR maintains stemness so that strategies can be identified to manipulate the Twist-EMT induced stem cell state in disseminated tumor cells by for instance, inducing differentiation or by enforcing long term permanent stemness resulting in therapeutic benefit. To accomplish this, we proposed 3 AIMS. We have made significant progress on the first two of these aims addressing the biochemical mechanism of Twist and its interaction partners at EMT response genes.

2. **KEYWORDS:** EMT, mesenchyme, differentiation, gene regulation, twist, E47, DNA binding protein, transcription, crystal structure, stem cell, stemness, metastasis, dissemination, dormancy, proteasome mediated protein degradation, E3 ligase activity, Snail, Zinc-fingers.

3. **ACCOMPLISHMENTS:**
What were the major goals of the project?

<u>**SOW Major Goals - % completed to date**</u>

Specific Aim 1: Define the role of candidate proteins bound by Twist WR in the induction of EMT and maintenance of the stemness phenotype, and search for novel proteins bound by WR in Twist induced stem cell populations.

95% completed

Specific Aim 2: Solve the structure of the WR domain when bound to the RUNX2 to determine how this interaction inhibits the binding of RUNX2 to DNA as a model for how Twist accomplishes this for other targets.

60% completed

Specific Aim 3: Define the role of the Twist bHLH and WR domains in induction of EMT, generation of cells with a stem cell phenotype and the maintenance of this phenotype using site directed mutagenesis of each domain in concert in vitro and in vivo cell models of EMT and stem cell generation.

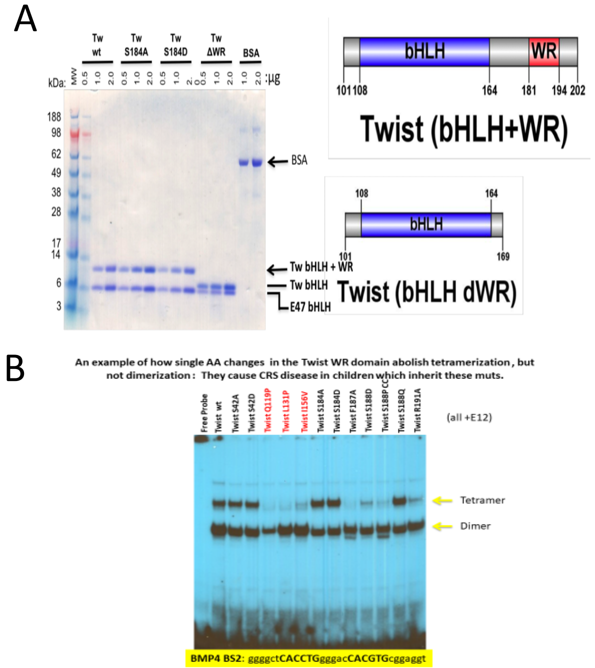
10% completed

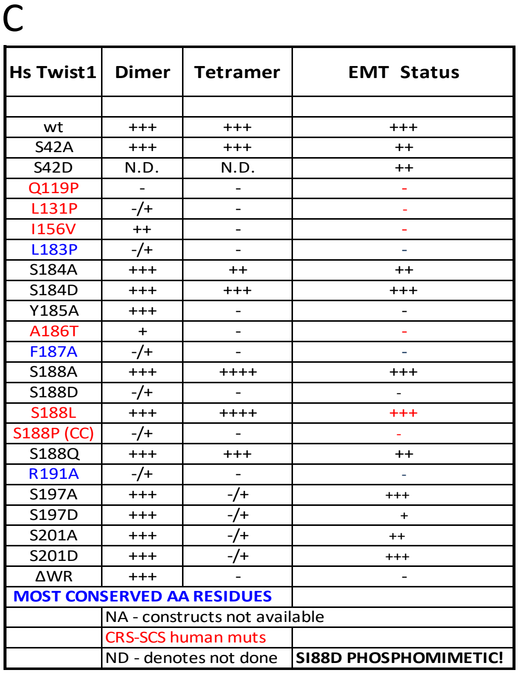
What was accomplished under these goals?

Accomplishments; By AIM in the SOW:

Specific Aim 1:
Define the role of candidate proteins bound by Twist WR in the induction of EMT and maintenance of the stemness phenotype, and search for novel proteins bound by WR in Twist induced stem cell populations.

Conserved amino acids in the WR domain has been subjected to site directed mutagenesis: most of the changes are to alanine and expected to change protein-protein interactions attributed to that AA/region of the protein. Each of these proteins have been expressed, purified, and used for DNA binding assays as shown in Figure 1 (also shown in last yearly report: presented here just for introduction and clarity). Although some of the mutant can be expressed at a detectable level, we were successful in purifying wildtype and 23 mutant proteins (Figure 1). As can be seen, a number of these single AA substitutions alter the ability to form a tetramer on DNA. These mutant proteins/genes were also tested for induction of EMT in SUM1315 and HMLE cells and for the ability to bind to Runx1 and inhibit lineage specific differentiation. Thus, AIM 1 supplied a large spectrum of site directed mutants which were used to explore the correlation between: 1. WR domain function, 3. tetramerization, 4. induction of EMT and inhibition of a lineage specific transcription factor (Runx2). Using this beautiful set of site-directed mutations in Twist-WR, we expected to find a correlation between these functions (listed 1-4, above). However, despite an exhaustive analysis of both wild type and mutant Twist-WR proteins with respect to these functions, we could not do so.

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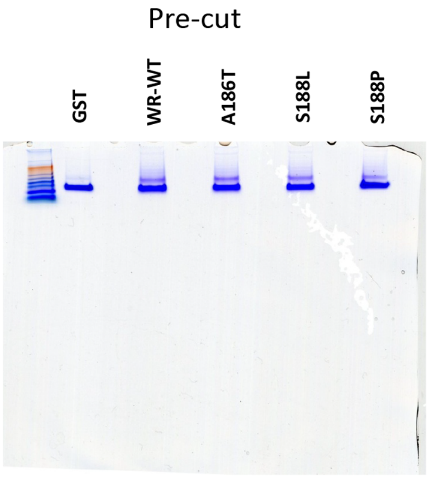
Hs Twist1	Dimer	Tetramer	EMT Status
wt	+++	+++	+++
S42A	+++	+++	++
S42D	N.D.	N.D.	++
Q119P	-	-	-
L131P	-/+	-	-
I156V	++	-	-
L183P	-/+	-	-
S184A	+++	++	++
S184D	+++	+++	+++
Y185A	+++	-	-
A186T	+	-	-
F187A	-/+	-	-
S188A	+++	++++	+++
S188D	-/+	-	-
S188L	+++	++++	+++
S188P (CC)	-/+	-	-
S188Q	+++	+++	++
R191A	-/+	-	-
S197A	+++	-/+	+++
S197D	+++	-/+	+
S201A	+++	-/+	++
S201D	+++	-/+	+++
ΔWR	+++	-	-
MOST CONSERVED AA RESIDUES			
	NA - constructs not available		
	CRS-SCS human muts		
	ND - denotes not done		**SI88D PHOSPHOMIMETIC!**

Figure 1. A. Biochemical purification of TWIST and its WR domain deletion mutant. B. Mutations in Twist can affect dimerization, tetramerization and DNA binding of the protein. Electrophoresis Motility Shift Assays (EMSAs) have been utilized with P^{32} γ-ATP labelled DNA probes to show the impact on binding that different mutations have on the double E-box transcriptional activation domain of a Twist1 target gene for EMT, BMP4. These mutations are derived from both developmental disease relevant mutations in patients and computational methods in the lab. Tetramerization of Twist1 and binding partner (E12 in this case) on the double E-box regulatory elements are necessary for activation of EMT and can be clearly distinguished from the less relevant dimerization. C, Summary of DNA binding, dimerization, tetramerization, and EMT phenotype for each of the TWIST mutations generated.

Proteomic analysis of Twist WT and Mutant complexes. New data is provided showing the results of a LC/MS/MS analysis of proteins that are associated with Twist wild-type (WT) or mutant proteins defective for EMT were isolated from stable cell lines SUM1315 and analyzed for associated proteins. The top hits are listed in **Fig. 2**.



Protein names	>10-fold lower In A186T vs WT	>10-fold lower In S188L vs WT	>10-fold lower In S188P vs WT
DNA repair protein RAD51 homolog 4	*	*	*
Metaxin-2			*
Cancer-related nucleoside-triphosphatase			*
Serine/threonine-protein kinase MST4			*
Lysophospholipase-like protein 1	*		
Transmembrane and coiled-coil domain-containing protein 1			*
Transmembrane emp24 domain-containing protein 1		*	
Transportin-3	*		*
Vesicle transport protein GOT1B			*
Deubiquitinating protein VCIP135		*	

Figure 2. Proteomic analysis of Twist complexes. Proteomics Methods: Samples were run into a gel for a short distance of 0.5 cm and stained with Coomassie blue. The entire stained gel regions were excised, digested in-gel with trypsin, and analyzed by LC-MS/MS on the ThermoScientific Q Exactive Plus mass spectrometer. MS data were searched against the UniProt human database using the MaxQuant 1.5.2.8 program. False discovery rates (FDR) for protein, and peptide were set at 1%.

Apart from the technical issues related to experimental feasibilities (unable to purifying some of the mutants), AIM 1 is 90 % completed.

Specific Aim 2: Solve the structure of the WR domain when bound to the RUNX2 to determine how this interaction inhibits the binding of RUNX2 to DNA as a model for how Twist accomplishes this for other targets.

For AIM 2, to map the interaction between Twist-WR and Snail, we used purified recombinant snail zinc finger (ZF) region proteins and truncations of Snail. As shown in Figure 3, Snail ZF region containing fingers 1-4 readily bound to GST-Twist-WR. In addition, the minimal interaction domain in the ZF region is comprised of fingers 2-4. Thus Snail, anchored by 3 of its 4 fingers binds with high affinity to Twist-WR.

As shown below, the Snail bound to Twist-WR likely functions as an E3 ligase for degradation of Runx2.

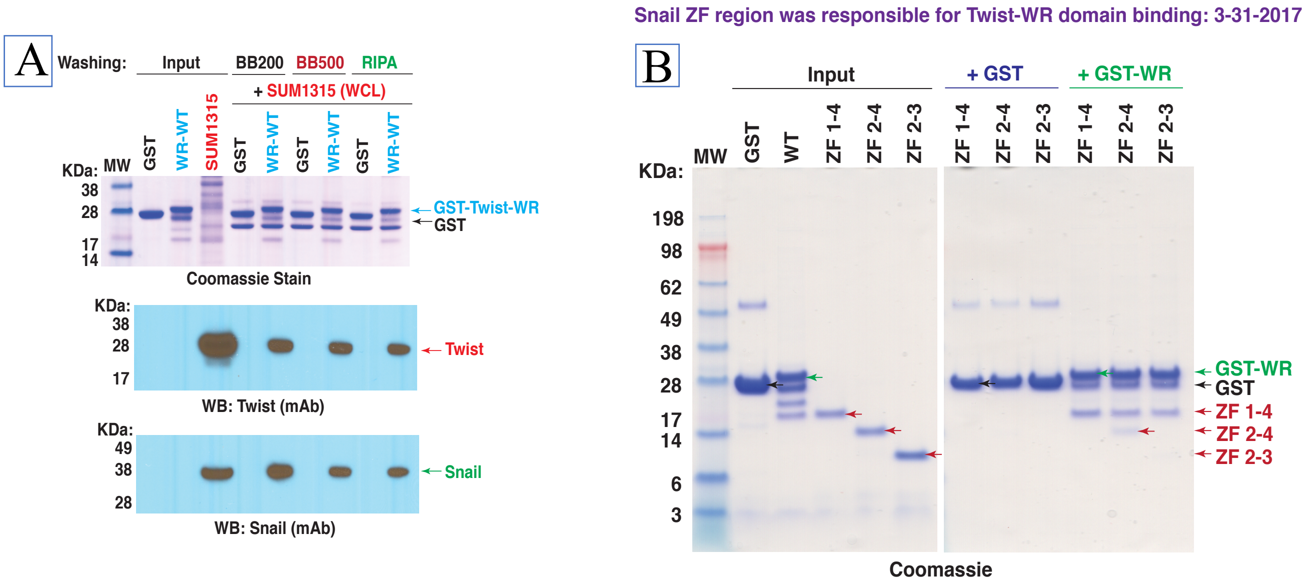


Figure 3: Panel A: SUM1315 cell derived Twist and Snail proteins readily bind to a GST-WR recombinant protein with high affinity. Panel B: Snail zinc fingers 2-4 are necessary and sufficient to bind to GST-WR.

In progress reported last year, we showed beautifully co-expressed, and purified (from E.coli) Twist-WR and E47 minimal proteins which we showed are highly functional in dimerization, tetramerization and binding to a DNA target site (Fig. 1). We also showed production and purification of recombinant Runt domain from Runx1 protein which is highly conserved with the Runt domain of Runx 2, and more amenable for recombinant protein production and structure determination. We used these protein preparations to reconstitute Runt domain DNA binding to its target site and its inhibition by Twist-WR (+E47). However, despite extreme effort using multiple preparations of protein, co-expression, and use of mammalian cell derived proteins, we were unable to show that Twist-WR could inhibit Runt domain of Runx1. An example of this data (all negative) is shown in Figure 3.

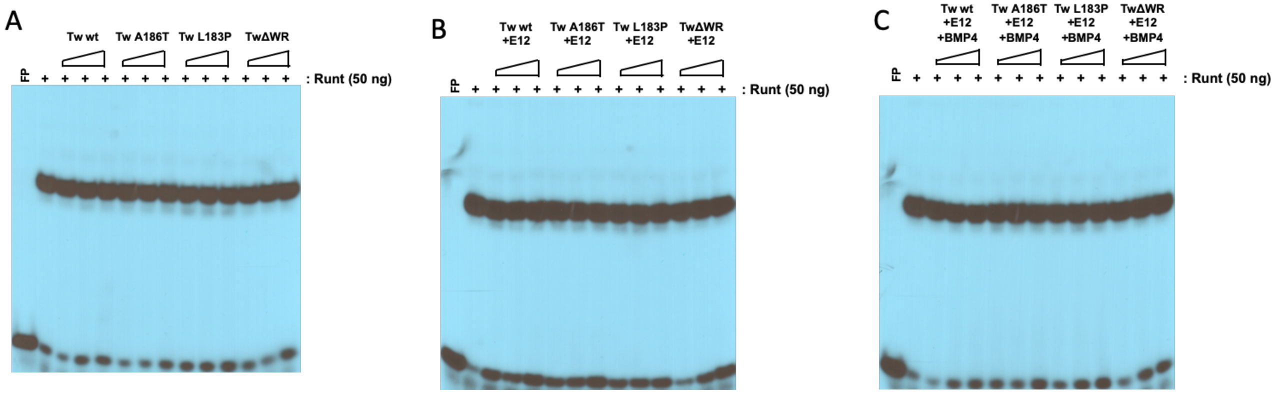


Figure 4. **Highly purified Twist-WR protein does not inhibit the DNA binding capability of Runt domain of Runx1**. For each lane, 50ng of highly purified Runx1 protein was used and a p32 labelled high affinity Runx binding site. Panel A: the indicated twist proteins alone were titrated into the binding reaction. Panel B: the indicated twist proteins plus the twist dimerization partner, E12 were titrated into the binding reaction. Panel C: The indicated twist proteins plus E12, plus the high affinity twist binding site from the BMP4 promoter were titrated into the binding reaction. Thus, twist alone, twist +E12 nor twist+E12+DNA complex does not inhibit Runx1 DNA binding.

Since we could show no inhibition of Runt domain binding to DNA, we turned to cell-based assays first to simply determine if there was any interaction at all between them, in a more physiologic environment. We transfected plasmid DNAs expressing full-length Twist-WR, Runx2 and Snail proteins into COS1 cells. After 48 hours, whole cell extracts were prepared, and proteins detected by western blotting.

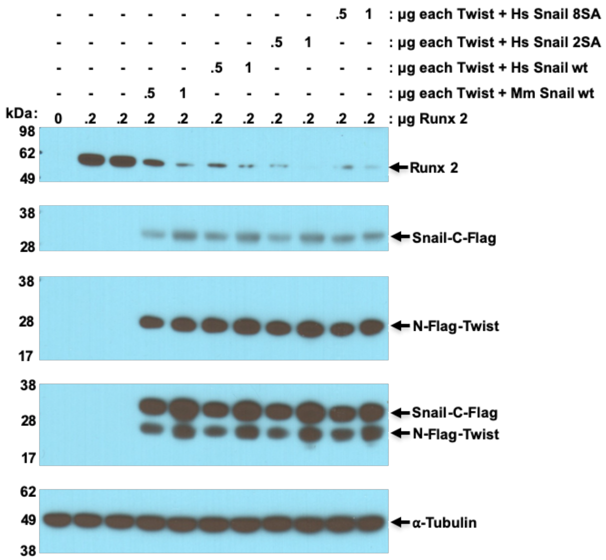


Figure 5. Twist plus Snail highly stimulate the degradation of Runx2 in cell culture systems. COS-1 cells were transfected with indicated plasmids. After 48 hours, lysates were prepared, electrophoresed on a 4-12% NuPAGE, transferred, and immunoblotted with indicated antibodies. Also, to control for loading, the blot was probed with α-Tubulin antibody. Untransfected cells (lane 1), transfected with Runx2 alone (lanes 2-3), transfected with Runx2 plus Twist and mouse Snail wt (lanes 4-5), transfected with Runx2 plus Twist and human Snail wt (lanes 6-7), transfected with Runx2 plus Twist and Human Snail 2SA mutant (lanes 8-9) and transfected with Runx2 plus Twist and Snail 2SA mutant (lanes 10-11). Snail 2SA is β-TRCP destruction box mutant while Snail 8SA is both GSK3β phosphorylation and β-TRCP destruction box mutant.

Remarkably, we discovered that twist and its high affinity partner snail, form a protein complex that targets Runx proteins (both Runx1 and 2) for degradation. Thus, the biochemical basis for twist inhibiting Runx1 thereby keeping cells in the stem cell like, un-differentiated state is not inhibition of Runx DNA binding. Rather, the mechanism is that twist + snail degrades Runx proteins in a very specific manner.

Future studies will be required to define the mechanism whereby twist + snail targets Runx 1 and 2 for degradation. We have shown that only the zinc finger region of snail is required to stimulate Runx2 degradation. This region overlaps with the ZF region of snail required to bind to twist-WR (Figure 2B). We now hypothesize that the snail ZF region functions as an E3 ligase for the degradation Runx proteins by the proteasome.

AIM 2 is 60% complete.

Specific Aim3. Define the role of the Twist bHLH and WR domains in induction of EMT, generation of cells with a stem cell phenotype and the maintenance of this phenotype using site directed mutagenesis of each domain in concert in vitro and in vivo cell models of EMT and stem cell generation.

Despite the efforts, only very limited progress has been made on AIM 3, which in large part due to complications in establishing the hormone-inducible cell lines. Accordingly, these technical difficulties will be resolved in the future studies.

What opportunities for training and professional development has the project provided?
Nothing to report.

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?
Due to the resignation of the PI, Dr. Frank Rauscher, from the Wistar Institute the project will be terminated after year 2. There is no request for a 3rd year of funding. The final aim of developing the hormone inducible cell line, and some of the stem-cell isolation assays have not yet been accomplished.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
We have established a unique protein co-expression system using both tagged and un-tagged proteins, all contained in a single plasmid DNA. This method should be very useful for other investigators to try. The co-expression of soluble active Twist-E47 complex has never been accomplished by others. We are the first to do it.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change
Due to the resignation of the PI, Dr Rauscher, from The Wistar Institute the project will be terminated early. The efforts to develop the hormone inducible cell line and isolation of stem cell phenotype have not yet been achieved.

Actual or anticipated problems or delays and actions or plans to resolve them
Problems of protein expression are described above in the narrative. They have been solved.

Changes that had a significant impact on expenditures
No Changes Required or anticipated.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

Significant changes in use or care of human subjects.
Nothing to report.

Significant changes in use or care of vertebrate animals.
Nothing to report.

Significant changes in use of biohazards and/or select agents.
Nothing to report.

6. PRODUCTS:

Publications, conference papers, and presentations
Nothing to report.

Website(s) or other Internet site(s)
Nothing to report.

Technologies or techniques
Nothing to report.

Inventions, patent applications, and/or licenses
Nothing to report.

Other Products
Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Frank J. Rauscher III, Ph.D.
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1.2
Contribution to Project:	Overall Supervision and Direction
Funding Support:	This award

Name:	Hongzhuang Peng, Ph.D.
Project Role:	Sr. Staff Scientist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	5.0
Contribution to Project:	Expression of Twist and E47 and RUNX
Funding Support:	This award

Name:	Dan McCracken
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2.0
Contribution to Project:	Dan has cloned, expressed and purified Twist-E47 complexes and has shown the twist complex in SUM1315 cells.
Funding Support:	This award and NIH T32 Cancer Biology Training grant

Name:	Kasirajan Ayyanathan, Ph.D.
Project Role:	Sr. Staff Scientist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	5.5
Contribution to Project:	Discovery of Twist degradation of Runx
Funding Support:	This award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report.

What other organizations were involved as partners?
Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES: N/A