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14. ABSTRACT

This Research Project was funded to define the roles of bacterial pathogen proteins which modify host proteins by post-translational modification. We have made great progress in accomplishing all of the AIMS of this project. The NF- B pathway is most critical for immune defense against infection, thereby frequently targeted by bacterial virulence effectors. NleE, an effector from EPEC and related enteric bacteria, is a SAM-dependent methyltransferase that blocks host NF- B-mediated inflammation. We have solved the crystal structure of NleE-SAM complex, which reveals a methyltransferase fold different from those of known methyltransferases. We further identify a new NleE substrate: ZRANB3, which has well defined roles in PCNA binding and remodeling of stalled replication forks at sites of DNA damage. NleE-catalyzed cysteine methylation of the ZRANB3-NZF domain also abolishes its K63-linked ubiquitin chain-binding activity, a key modification of PCNA which initiates repair. Specific inactivation of the NZF domain in ZRANB3 by NleE, and hence its DNA repair functions suggests a novel and unexpected link between EPEC infection, virulence proteins and genome integrity. We have also discovered a new NleE substrate: ZRANB1/ Trabid1 , which contains three target zinc fingers and differentially methylates these three fingers to modulate binding to mixed ubiquitin linked chains. Trabid1 functions in the NF-kB pathway in innate immunity like TAB2/3.

15. SUBJECT TERMS

SAM : S-Adenosyl Methionine, NleE: the NleE Effector Protein, EPEC: E.Coli Effacing Bacteria

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1. INTRODUCTION:

NF-κB signaling plays a central role in defending against bacterial infection. Upon infection, the NF-κB signaling initiates innate immune response and inflammation in response to recognition of (pathogen-associated molecular pattern) PAMP molecules by membrane-bound Toll-like receptors or other intracellular pattern-recognition receptors. The NF-κB signaling can also be activated by various cytokines such as TNFα and IL-1β upon their ligation of the corresponding receptors on the plasma membrane [1] . These receptors complexes generate ubiquitin-chain signals that are directly sensed by the TAB2/3 adaptors, which further activate the TAK1 and IKK kinase cascade. Consequently, the NF-κB transcription factors are released from the cytosol into the nucleus and initiate transcription of genes related to immune defense. Given the paramount role in counteracting pathogen infection, the NF-κB signaling is frequently targeted by bacteria or bacteria-secreted toxins. For EPEC and the related enterohemorrhagic *E. coli* (EHEC), several bacterial proteins that are injected into host cells by the conserved bacterial type III secretion system (T3SS) have been identified by virtue of their biochemical activity of blocking the host NF-κB signaling. Genetic analyses of these effectors function suggest that the NleE effector, which is also conserved in *Shigella* and certain *Salmonella* species, plays a major role in EPEC/EHEC suppression of the NF-κB signaling in infected cells in culture. In a previous study it was discovered that NleE harbors an unprecedented SAM-dependent methyltransferase activity [2]. NleE specifically targets a zinc-coordinating cysteine in the NZF domains of TAB2/3 for methylation modification. NleE-catalyzed cysteine methylation results in departure of the zinc ion and loss of the ubiquitin chain-binding ability of TAB2/3 NZF domains, thereby blocking the NF-κB-mediated proinflammatory responses during bacterial infection.

This project funded by DOD was designed to further explore the substrates and biological functions of NleE during host infection. Protein methylation is of great importance in a plethora of cellular processes including biosynthesis, signal transduction, protein repair, chromatin regulation and gene silencing. In methylation modification, methyltransferase transfers a methyl group from a donor onto a substrate acceptor using versatile catalytic strategies. SAM is the most commonly used methyl donor for known methyltransferases. SAM-dependent methyltransferases are highly diverse in their primary sequence, three dimensional structure and SAM-binding mode, and have been structurally classified into five different families (Class I-V). The majority of known protein methyltransferases belong to the Class I and Class V, and other classes of methyltransferases are restricted to targeting DNA, RNA and small molecules for methylation modification.

Protein methylation generally occurs on a lysine or an arginine side chain, as have been best illustrated in histone methylation in epigenetic regulation of gene transcription. In addition to *N*-methylation in lysine and arginine, thiol methylation has also been recorded. The *E. coli* sacrificial DNA repair protein Ada accepts a methyl group from methylated DNA in a non-enzymatic manner, thereby conferring cellular resistance to genotoxic stress by methylating agents. Betaine-homocysteine *S*-methyltransferase and methionine synthases can catalyze thiol methylation on a small molecule substrate (homocysteine), and function in homocysteine metabolism and

methionine biosynthesis, respectively. NleE-catalyzed methylation of TAB2/3 represents the first example of enzyme-catalyzed protein cysteine methylation, representing a novel mechanism in regulating signal transduction in eukaryotes. Notably, in all three cases of thiol methylation noted above, the methyl group acceptor is one of the four cysteine/homocysteine residues coordinating a zinc ion. Zinc coordination by itself can increase the nucleophilicity of a thiol, therefore facilitating the presumed S_N2 nucleophilic attack on the donor methyl group. NleE methylation differs from the other two types of thiol methylation in using SAM as the methyl donor. In fact, NleE harbors no sequence homology to all known methyltransferases and is the first SAM-dependent methyltransferase that modify a cysteine. However, the structural basis of NleE SAM-dependent methyltransferase activity and mechanism underlying NleE substrate specificity for a zinc-finger cysteine in TAB2/3-NZF domains were largely unknown. Thus we undertook the approved project to decipher how NleE worked as an enzyme, and to define its regulation in infected cells.

2. KEYWORDS:

S-ADENOSYL METHIONINE
PROTEIN METHYLATION
ZINC FINGER
BACTERIAL VIRULENCE PROTEINS
EPEC BACTERIA
CYSTEINE METHYLTRANSFERASE
ARGININE METHYLTRANSFERASE

3. ACCOMPLISHMENTS:

What were the major goals and objectives of the project?

OBJECTIVES: We hypothesized that NleE is a first in class, bacterially-encoded virulence effector protein that targets DNA repair pathways in the host colon cell that can directly influence genome stability during colon cancer progression. Virtually nothing is known of the dynamics of NleE protein function in colon cells and how methylation of its substrates can influence cell growth and tumor progression. We will test this hypothesis by performing the following Specific Aims:

AIM1: Define the regulation and dynamics of NleE function *in vivo* in colon cancer cells during EPEC infection, the protein complexes in which it resides, and the intracellular response in colon cells to this protein.

AIM2: Characterize the ZRANB3 and NPL4 C4 ZF proteins as novel NleE substrates, define their role in DNA repair pathways in colon cancer cells and the consequences of their inactivation by cysteine methylation to the genomic stability of the colon cells.

What was accomplished under these goals?

The original objectives as described in the AIMs above were changed during the course of the project due to both technical difficulties and to changes in research direction warranted by the results obtained. This has taken place as the normal ebb and flow of research occurring in a fast changing laboratory environment and dictated by the ability to make critical reagents. These changes will be directly addressed below where progress on the individual elements of the original SOW are indicated. A revised SOW based on the work accomplished and envisioned is provided at the end of this section.

A quick summary the major changes which have come about:

AIM1: Instead of defining the regulation and cellular binding partners of NleE in the infected cell, we shifted our emphasis to defining the molecular structure of the enzyme while it is bound to its obligate co-factor SAM. We succeeded in defining the first high resolution crystal structure of a SAM dependent cysteine directed methyltransferase. This change in focus was because it was very difficult technically to make antibodies to NleE for tracking it in the cell and its complexes. However, the recombinant NleE was extremely robust when derived from E.coli and was amenable to structural characterization so we solved its crystal structure.

AIM2. Instead of following up the NPL4 zinc finger protein as a novel NleE substrate, we discovered an alternate, more robust and interesting substrate, ZRANB1/Trabid1. As described in unpublished data shown below, its highly likely Trabid1 regulates the NFkB pathway as do the other substrates of NleE. As we initially proposed, we also have thoroughly characterized ZRANB3 as a novel substrate of its enzyme activity. So in summary, in completion of this AIM2, we still have characterized 2 completely novel substrates for NleE.

Progress on each Specific Proposed AIM and sub-AIM in original the SOW:

From the original SOW

Specific Aim 1: Define the regulation and dynamics of NLEE function *in vivo* in colon cancer cells during EPEC infection, the protein complexes in which it resides, and the intracellular response in colon cells to this protein.

Subtask 1: Establish stable NLEE expressing colon cancer cell lines:

PROGRESS: Despite many efforts, it proved impossible to generate stable cell lines expressing NleE. We tried this with both constitutive and inducible viral and plasmid based vectors. All of the cells died. It is well known that most cultured cells cannot remain viable without an intact NFkB system. We suspect that even with our most tightly controlled inducible systems, the leakiness of this very powerful repressor of N9FkB function led to cell death.

Subtask 2: Characterize and biochemically purify a complex of proteins that associate with NLEE from the cell lines.

PROGRESS: Despite superbly expressed and purified NleE from E. coli, we could not raise suitable rabbit antibodies to NleE. The protein may have a very low antigenicity. These abs are crucial to looking at endogenous NleE complexes in infected cells. Thus we were unable to do these experiments. Even with transient transfection of NleE cDNAs and using tagged constructs, we were unable to robustly identify protein complexes. This was quite a lot of work which did not come to fruition.

Subtask 3: Identify the proteins binding to NLEE using MS/MS, obtain CDNAS, express proteins and determine if they are substrates and/or modulators of NLEE function.

PROGRESS: As described above, we could not generate the tools required (cell lines, antibodies) to complete a full identification of NleE binding proteins.

After struggling with the original AIMS described above, we switched to using the robustly made and highly enzymatically active NleE to solve its crystal structure and perform a full structural biology based study. This was highly successful as described below;

A unique structure for SAM-dependent methyltransferases:

This work was published by us in collaboration Dr. Feng Shao in a landmark paper [3] :

Yao Q, Zhang L, Wan X, Chen J, Hu L, Ding X, Li L, Karar J, Peng H, Chen S, Huang B, Rauscher FJ III, Shao F. 2014. ***Structure and specificity of bacterial cysteine methyltransferase effector NleE suggests a novel substrate in human DNA repair pathway****. PLoS Pathog.* 10(11).

The experimental strategy, comprehensive methods and results are contained in that paper, attached to this report. Below, I will summarize the most salient points and refer to the numbered figures in that paper [3].

In lieu of the technical difficulties in performing much of AIM1 in the original SOW, we sought to capitalize on the robust nature of recombinant NleE by solving its structure [3]. We discovered that as a SAM-dependent methyltransferase, NleE is unique in catalyzing cysteine methylation. The structure of NleE bears an overall Rossmann-like fold and more resembles that of Class I methyltransferase among the five classes of SAM-dependent methyltransferases (Figure 2) However, the SAM-binding mode and the conformation of the SAM moiety in NleE are completely different from Class I and other known methyltransferases. Furthermore, NleE bears neither GxGxG-containing motif nor any sequence similarity to Class I methyltransferase (Figure 4A). This indicates an independent evolution of the two sub-lineages within the large

methyltransferase family and highlights the convergent evolution of bacterial virulence activity. The NleE structure represents a novel methyltransferase fold. The amino acids R107, E191 and Y212 at the bottom of a deep and narrow cavity in NleE chelate and position the SAM into a distinct conformation ready for nucleophilic attack by the substrate (Figure 2). The cavity can accept and well hold the incoming substrate NZF domain and molecular dynamic simulation of the docked NleE-SAM-NZF complex indicates that Cys673 in TAB2-NZF is structurally and energetically favorable for attacking the SAM (Figure 3). The key amino acids in the active site for both chelating SAM and for catalysis were identified by comprehensive site directed mutagenesis (Figure 3C).

Methylation usually occurs on a lysine or an arginine side chain and cysteine methylation has been rare in published literatures. We think that chemically, zinc binding increases the nucleophilicity of the cysteine thiol, enabling the nucleophilic attack onto the methyl donor through the S_N2 mechanism. These findings further support the notion that zinc coordination could facilitate methyl transfer onto the cysteine thiol. Given that the small zinc finger motif is highly abundant in a given proteome it is reasonable to expect the existence of other zinc-finger motifs as potential methylation targets.

Thus our work, supported by this DOD grant on the structure of NleE provides a first snapshot of SAM-dependent cysteine methyltransferase distinct from those characterized ones for lysine or arginine modification. Identification and characterization of NleE methyltransferase activity expands the repertoire of SAM-dependent methyltransferases and highlights the convergence on methylation chemistry from different three dimensional folds. In additional work described below, we discovered and characterized two different substrates for cysteine methylation by NleE.

From original SOW:

Specific Aim 2: Characterize the ZRANB3 and NPL4 C4 ZF proteins as novel NLEE substrates, define their role in DNA repair pathways in colon cancer cells and the consequences of their inactivation by cysteine methylation to the genomic stability of the colon cells.

Subtask 1: Determine if NLEE transfection alters the ability of ZRANB3 or NPL4 to be rapidly recruited to DNA replication forks after DNA damage.

PROGRESS: We first characterized ZRANB3 as a robust and unique substrate for methylation by NleE. This work was published in the article cited above [3] and the salient points are summarized here again with reference to the figures in the paper.

We profiled almost every C4 zinc finger protein encoded in the human proteome. The C4 finger coding regions were individually isolated, cloned and expressed as recombinant proteins in E. Coli and then tested for methylation by recombinant NleE. Of the 50+ C4 fingers we expressed, the most robust substrates were ZRANB3 and

ZRANB1/Trabid1 (supplementary data [3]). While the NPL4 finger showed demonstrable methylation, it was an order of magnitude less well methylated than the above C4 ZFs. For this reason, we moved forward with these two substrates.

ZRANB3 as a new host target of NleE cysteine methyltransferase effector

Profiling of the NZF domain-containing proteins identify a large multi-domain protein ZRANB3 as a new substrate of NleE (Figure 7). We have also discovered that NleE can methylate the NZF domain of ZRANB3 with efficiency comparable to that of TAB2/3-NZF domains (Figure 7). ZRANB3-NZF can readily bind to all three major linkages of ubiquitin chains (K48, K63 and linear) and its methylation by NleE results in a complete loss of the ubiquitin chain-binding activity. However, NleE-induced functional loss of the NZF domain in ZRANB3 does not affect ZRANB3 recruitment to the DNA damage site (Supplementary data [3]). Previous studies indicate that proper function of ZRANB3 strictly depends on its interaction with PCNA and three domains, including the PCNA-interacting protein motif (PIP-box), the C-terminal AlkB2 PCNA-interaction motif (APIM) as well as the NZF domain, are proposed to mediate this interaction [4]. ZRANB3-NZF can bind to K63-linked poly-ubiquitinated PCNA, which is believed to be important for ZRANB3 recruitment to DNA damage sites. Using NleE as a unique probe, we could inactivate the NZF domain without interfering with other domain functions in ZRANB3, which instead reveals a dispensable role of the NZF domain in DNA damage recruitment. Thus, the NZF domain either does not play a role in the recruitment process or is functionally redundant to other domains. The latter hypothesis appears to be supported by data showing that the PIP-box or APIM mutant of ZRANB3 are more attenuated than the NZF-domain mutant in being recruited to DNA damage sites [4].

The activity we have discovered for NleE offers an unprecedented opportunity for functional inactivation of the NZF domain in ZRANB3 *in situ*. Thus, it is more plausible that NZF domain-mediated binding to poly-ubiquitin chains (possibly on PCNA) may instead regulate the activity of ZRANB3 itself or fulfill functions other as yet undefined functions of ZRANB3. However, the concept, suggested by these results; that EPEC infection could directly contribute to the genetic instability of infected colonocytes is interesting to contemplate. It is noteworthy that MIN mice, which are predisposed to polyps and frank colon tumors, develop increased numbers and larger, more aggressive tumors when they are infected with EPEC bacteria. These structural and functional analyses further indicate that NleE may target ZRANB3 or other zinc-finger proteins for cysteine methylation in promoting bacterial virulence.

ZRANB1 as a new host target of NleE cysteine methyltransferase effector

Since our survey indicated that ZRANB1/Trabid was also a robust substrate for NleE in vitro, we further characterized it. Interestingly, ZRANB1/Trabid is a deubiquitinating (DUB) enzyme, with some sequence similarity to A20, another very important DUB, negatively regulating the NF-kB pathway [5]. ZRANB1/Trabid has three C4 zinc fingers in its N terminal region [5] through which it can bind to ubiquitin chains and it cleaves these chains via a catalytic OTU (ovarian tumor) domain [6]. It was found

that ZRANB1/Trabid can deubiquitinate K63 linked polyubiquitin chains very efficiently and it could deubiquitinate APC protein, thereby positively regulating the Wnt signaling pathway . Remarkably it was later observed that ZRANB1 deubiquitinates atypical ubiquitin linkages, with high preference to K29 and K33 linked ubiquitin [7]. Recent studies have provided in depth structural insights into the binding of K29 di-ubiquitin ya[8] and K33 di-ubiquitin [9] with the ZF1 region of ZRANB1 suggesting that each separate finger may bind a different type of Ub linkage.

In this study, we started off by investigating whether NleE methylates all the three zinc fingers of ZRANB1 or not and what effect of this methylation has on the ubiquitin binding and the DUB activity of ZRANB. We showed that (see figures below) that NleE differentially methylates the three ZFs, however we have yet to show if this modification modulates activity in vivo (ongoing work). The methods used for the studies below were identical to the ones used in our published paper [3]. The data shown below is ALL UNPUBLISHED.

NleE differentially methylates ZRANB1 ZFs

The Zinc finger regions of all of the known substrates for NleE are shown in Figure 1. As expected, in addition to the regularly spaced cysteine residues, they show significant but far from identical primary amino acid sequence (Figure 1). We purified the three zinc fingers of ZRANB1 either individually (ZF1, ZF2 and ZF3) or together (ZF1-3) and tested them in an *in vitro* methylation reaction using recombinant NleE and ^{3}H-S-adenosyl-L-methionine (^{3}H-SAM) (Figure 2). We observed that although the complete zinc finger region of ZRANB1 (GST-ZRANB1 ZF1-3) was methylated, it was a much weaker substrate of NleE compared to the more robust substrates TAB2-ZF and ZRANB3-ZF (Figure 2). Interestingly, when we compared the methylation of the three zinc fingers individually, we found that they were methylated differentially. The ZF3 region was methylated the most, followed by weak methylation of ZF1. The ZF2 was not methylated at all in vitro (Figure 2). Thus the fingers in ZRANB1/Trabid are not identical with respect to reactivity with NLeE.

Fig. 1 **Alignment of amino acid sequence of the C4 zinc finger regions of Tab2/3, Zranb3, Npl4 and Zranb1**

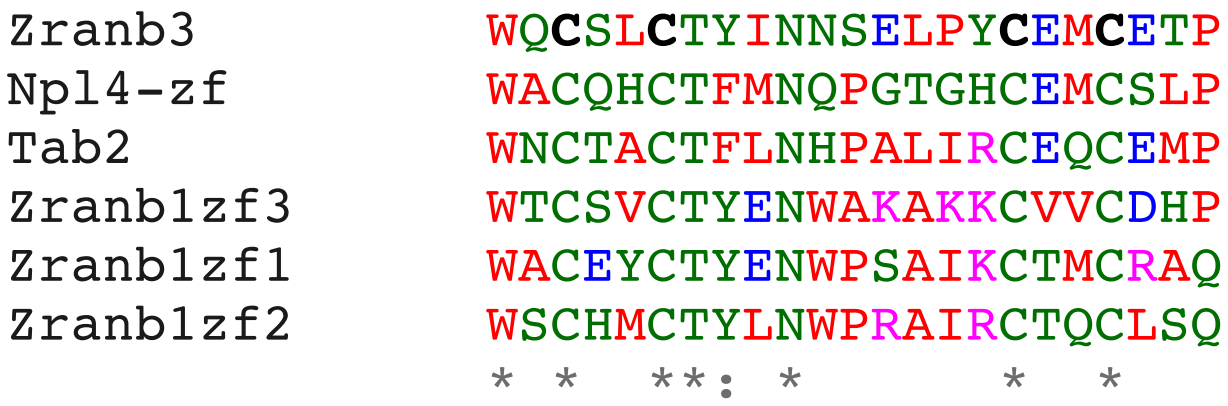

Figure 1: Alignment of the C4 zinc finger regions of each of the substrates of NleE. The canonical cysteine residues are in bold, black in Zranb3. The asterisks are completely conserved residues in the finger structure.

In vivo methylation of ZRANB1

We next wanted to study the methylation of ZRANB1 purified from mammalian cells. We transfected 293T cells with Flag ZRANB1 or Flag ZRANB3 for comparison, followed by flag pull down and *in vitro* methylation using purified NleE and ^{3}H-SAM. While NleE methylated ZRANB3 as expected, we observed a very weak signal for ZRANB1 (after a prolonged exposure), again suggesting that ZRANB1 is a weak substrate of NleE (Fig 2, lanes 5 and 2). The effect of *in vivo* re-methylation of ZRANB1 (and ZRANB3) by NleE was next examined. We co-transfected Flag ZRANB1 with HA-NleE–wild type or the mutant HA-NleEΔ6 (catalytically dead form of NleE with a deletion of 6 amino acids $_{209}$IDSYMK$_{214}$). We did a flag pull down and performed *in vitro* methylation using purified NleE and ^{3}H-SAM. In cells co-transfected with ZRANB1 and wild type NleE, ZRANB1 was not re-methylated by purified NleE. But, in cells co-transfected with mutant NleEΔ6, ZRANB1 was re-methylated but giving a weak signal, very similar to Flag ZRANB1 alone (Figure 3, lanes 5, 6 and 7). In cells co-transfected with ZRANB3 and wild-type NleE, recombinant NleE did not remethylate ZRANB3. However, ZRANB3 co-transfected with mutant NleE was efficiently methylated by recombinant NleE (Figure, lanes 3 and 4), suggesting full methylation of cellular ZRANB3 by transfected wild type NleE. Together these results show that both ZRANB3 and ZRANB1/Trabid are methylated inside cells which are transfected with catalytically active NleE.

Methylation of zinc fingers of Tab2, Zranb3, Npl4 and Zranb1 by NleE

Fig. 2

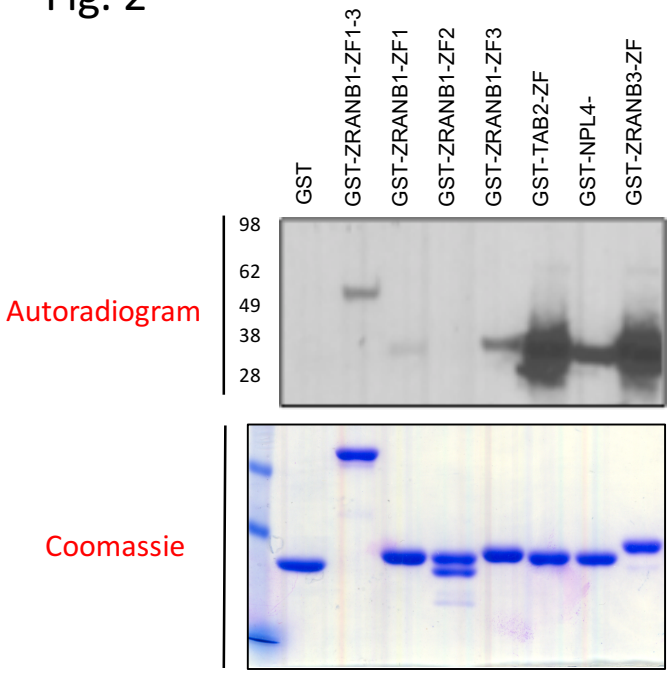

Figure 2: NleE differentially methylates each of the fingers aligned in Figure 1. The indicated purified recombinant proteins (coomasie stained gel) were incubated with recombinant NleE and 3H-SAM.

In vivo methylation of Zranb3 and Zranb1

Fig.3

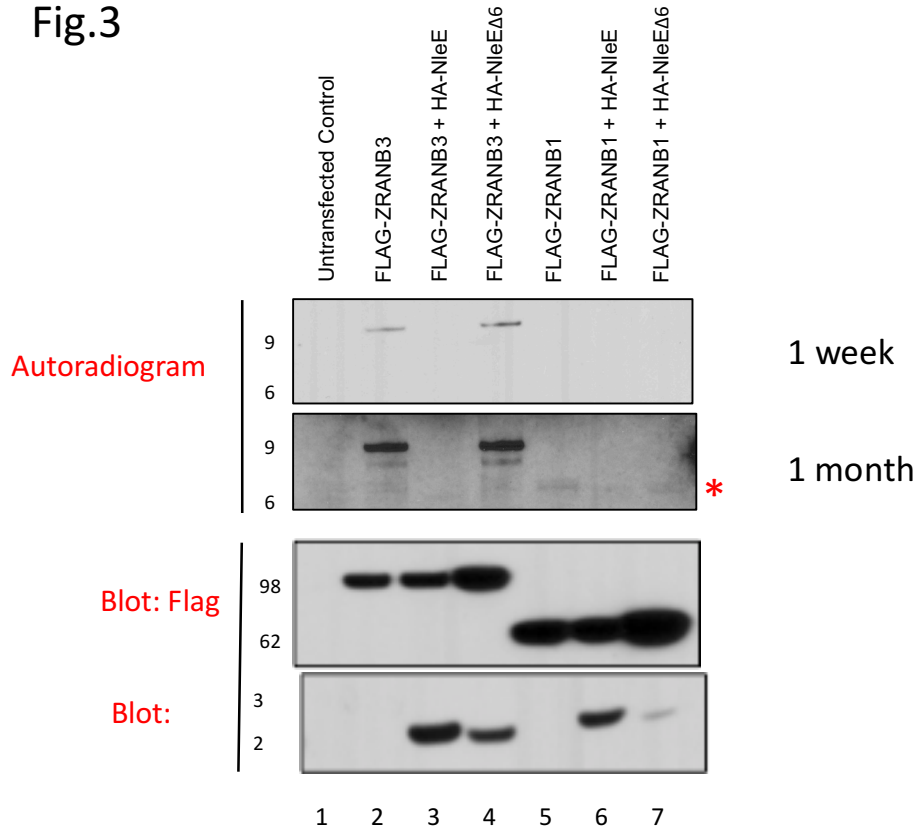

Figure 3: The NleE substrates ZRANB3 and ZRANB1/Trabid are methylated when transfected into cells. Expression vector DNA containing the full length cDNAs were transfected plus or minus NleE into 293T cells. The proteins were purified by immune precipitation and then subjected to a second round of in vitro methylation with NleE. If the protein isolated from cells cannot be re-methylated in vitro, this is good evidence that it has been robustly methylated in vivo.

Binding of ZRANB1 with polyubiquitin chains or diubiquitin

The three tandem zinc fingers of ZRANB1 can very efficiently bind to K63-linked ubiquitin chains [6]. We also found it to be the case when we checked for binding of K63 polyubiquitin chains with purified GST-ZRANB1 zinc finger region (GST-ZRANB1 ZF 1-3). The next obvious question we had was whether the methylation of the zinc fingers, albeit weak, affects the robust binding to K63 polyubiquitin. We first methylated the contiguous fingers ZF1-3 of ZRANB1/Trabid region using NleE and SAM in vitro and then checked for K63 polyubiquitin binding. A protein containing all three fingers bound to K63 poly Ub robustly. Moreover, in vitro methylation of that significantly inhibited binding (compare the last two lanes of Figure 3). Interestingly, when we checked each individual ZF expressed as a GST fusion, we observed no binding whatsoever to K63

Linked Ub (Figure 4, lanes 1-6). Also methylation obviously had no effect on polyubiquitin binding ability of the individual ZFs as well to bind. Thus, this suggests that each the three fingers of ZRANB1/Trabid cooperate in recognizing K63 linked chains. This is quite different than the case of the single finger modules of ZRANB3 and TAB2/3 which robustly bind K63 linked Ub as single finger units [2]! Sorting out the amino acid code in each finger required for binding K63 linked Ub will be facilitated by the data we have generated with these sets of fingers.

Finger 3 of ZRANB1/Trabid mediates K63 linked Ub binding: inhibition my NleE methylation in vitro

Fig.4

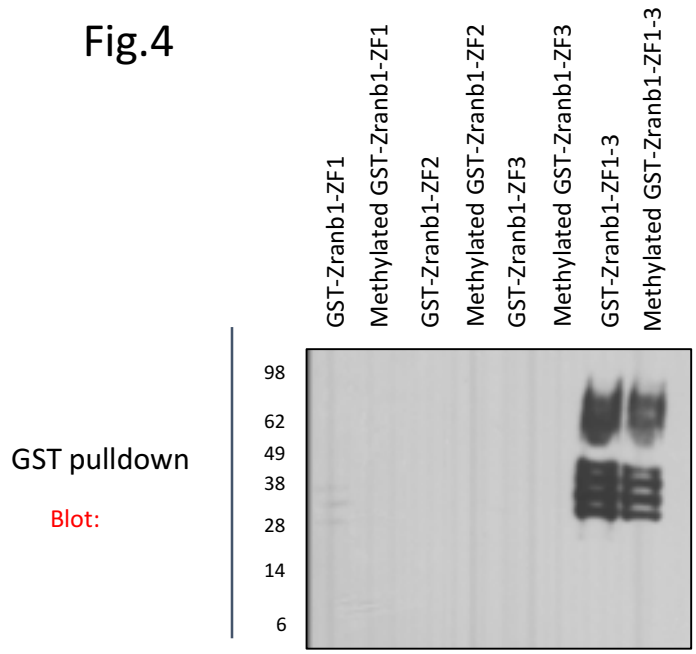

Figure 4: The three finger unit from ZRANB1/Trabid binds K63 linked Ub chains robustly. The proteins shown in the coomasie gel in figure 2 were incubated plus or minus NleE and then a mixture of purified K63 linked Ub chains were added and binding assessed by GST pulldown. Only the protein containing a full set of three ZF units bound to the Ub chains. Moreover, methylation reduced but did not abolish Ub binding (compare last two lanes).

Recent studies [9] [10]have reported some very interesting observations on ZRANB1/Trabid ability to bind to atypical ubiquitin especially K29 and K33 linked poly-ubiquitin. We also checked the ability of the purified Zranb1 ZF1-3 region to bind to K27, K29, K33 or K63 linked di-ubiquitin. Like previously reported we also observed that ZRANB1 ZF region binds to K29 and K33 di-ubiquitin (data not shown). Moreover, and very surprisingly, the three fingers of ZRANB1/Trabid did not bind to K27 or K63 linked di-ubiquitin (data not shown), showing the specificity of the ZF region to bind only

certain length chains of ubiquitin. Simply put, the three ZF module of ZRANB1/Trabidcan bind to ubiquitin chains of a certain length since it can bind to higher forms of K63 polyubiquitin but not K63 di-ubiquitin, another interesting observation which will be important to sort out in future studies.

In summary we have found the ZRANB1/Trabid is another substrate for NleE and that its three fingers are differentially methylated, likely resulting in the ability to bind different subsets of ubiquitin linked chains. The biological relevance of these observations are underscored and supported by a very recent paper [11] which shows that ZRANB1/Trabid regulates IL12 and IL23 during autoimmune responses in innate immunity. Thus it is reasonable that EPEC bacteria use NleE to target both TAB2/3 and ZRANB1/Trabid to reduce the innate immune response to infection in the gut. Whether or not ZRANB1/Trabid also has a role in DNA repair, like we hypothesized for ZRANB3, has yet to be determined.

Subtask 2: Knockdown endogenous ZRANB3 and NPL4 and reconstitute with an RNAi-resistant ZRANB3-GFP and NPL4-GFP fusion genes: track these proteins dynamically by live cell confocal imaging after NLEE transfection.

PROGRESS: Because we could not demonstrate that ZRANB3 was altered in localization to damaged DNA in the presence or absence of NleE, we refocused our efforts on the new and novel NleE substrate ZRANB1/Trabid,

Milestone(s) Achieved: Define the role and dynamics of NLEE at the replication fork and how it can alter DNA repair. Publish one-two papers in peer-reviewed journals

PROGRESS: 1 paper has been published [2] and one is being prepared for submission:. The title is: The bacterially encoded Cysteine Methlytransferase NleE virulence protein targets ZRANB1/TRABID, an OTU domain DUB that regulates PCNA ubiquitination.

Revised SOW Statement of Work (Accomplished):

AIM1: Purify and characterize recombinant NleE protein. Determine its biochemical and biophysical properties as an enzyme and solve the crystal structure of the enzyme with its co-factor SAM.

Subaim 1: Produce recombinant NleE

PROGRESS ; 100% completed

Subaim 2: Crystallize the NleE protein and subject it to Xray crystallographic structure determination.

PROGRESS: 100% accomplished

Subaim 3: Prepare a structural model of NleE, predict catalytic mechanism, and substrate binding regions and test these by site directed mutagenesis.

PROGRESS: 100% completed

AIM2: Perform a survey for potential substrates of NleE using recombinant C4 zinc finger proteins. Identify the best substrates and the sequence of their respective finger regions. Characterize these substrates for differential methylation by NleE in vitro and in transfected cells. Determine that effect of NleE methylation on the ability to bind to various ubiquitin chain lengths and linkages.

Subaim 1: Screen recombinant C4 ZFs to find the cellular substrates (ZRANB1/Trabid and ZRANB3)

PROGRESS: 100% accomplished

Subaim 2: Test in vitro and in vivo function of the substrates in the presence or absence of NleE.

PROGRESS: 50% accomplished. We still need more study to determine how the functions of both substrates are modulated by NleE to alter the proteins function in DNA repair (ZRANB3) and innate immunity (ZRANB1/Trabid)

Milestones Achieved: Publish two papers in peer-reviewed journals.

PROGRESS: 50% accomplished: One full length paper published [3], another one in preparation for which all the data are already collected.

What opportunities for training and professional development did the project provide?
Nothing to report.

How were the results disseminated to communities of interest?
These discoveries were reported in the following paper:

Yao Q, Zhang L, Wan X, Chen J, Hu L, Ding X, Li L, Karar J, Peng H, Chen S, Huang N, Rauscher FJ 3rd, Shao F. Structure and specificity of the bacterial cysteine methyltransferase effector NleE suggests a novel substrate in human DNA repair pathway. PLoS Pathog. 2014 Nov 20;10(11):e1004522. doi: 10.1371/journal.ppat.1004522. eCollection 2014 Nov. PubMed PMID: 25412445; PubMed Central PMCID: PMC4239114.

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

We will continue to characterize the in vivo role of both NleE substrates ZRANB3 and ZRANB1/Trabid in the remaining time and resources allotted. In the short term we will complete and submit the ZRANB1/Trabid paper for publication.

4. IMPACT:

Pathogens often manipulate host functions by posttranslational modifications such as ubiquitination and methylation. The NF-κB pathway is most critical for immune defense against infection, thereby frequently targeted by bacterial virulence effectors. The impact we have had is to show that NleE, an effector from EPEC and related enteric bacteria, is a SAM-dependent methyltransferase that blocks host NF-κB-mediated inflammation. NleE is not homologous to known methyltransferases and modifies a zinc-finger cysteine in TAB2/3 in the NF-κB pathway. We present the crystal structure of SAM-bound NleE that adopts α/β doubly-wound open-sheet architecture. The structure represents a novel methyltransferase fold with a unique SAM-binding mode. Further structural analyses, together with computational substrate docking and molecular dynamics simulation, illustrates a structural and chemical mechanism underlying NleE recognition of the NZF and catalyzing site-specific cysteine methylation. These results reinforce the idea of harnessing bacterial effectors as a tool for dissecting eukaryotic function and also indicate the presence of other zinc-finger proteins as the host target of NleE effector. This work has had impact on multiple fields by expanding the range of substrates and biological processes that can be manipulated by infective bacteria.

What was the impact on the development of the principal discipline(s) of the project?
Nothing to report.

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

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

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

5. CHANGES/PROBLEMS:

Nothing to report.

6. PRODUCTS:

Journal publications.
Yao Q, Zhang L, Wan X, Chen J, Hu L, Ding X, Li L, Karar J, Peng H, Chen S, Huang N, Rauscher FJ 3rd, Shao F. Structure and specificity of the bacterial cysteine methyltransferase effector NleE suggests a novel substrate in human DNA repair pathway. PLoS Pathog. 2014 Nov 20;10(11):e1004522. doi: 10.1371/journal.ppat.1004522. eCollection 2014 Nov. PubMed PMID: 25412445; PubMed Central PMCID: PMC4239114.

Books or other non-periodical, one-time publications.
Nothing to report.

Other publications, conference papers, and presentations.
Presented platform talk at the 2015 AACR Annual meeting in Washington, DC on the topic of NleE and colon cancer.

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:

Name:	Frank J. Rauscher, III Ph.D.
Project role:	Principal Investigator
Research Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2
Contribution to Project:	Coordinated project, wrote manuscript, supervised all investigators.

Name:	Hongzhuang Peng, Ph.D.
Project role:	Sr. Staff Scientist
Research Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3

Contribution to Project:	Purified the NLEE protein performed methylation assays.

Name:	Jayashree Karar, Ph.D.
Project role:	Assoc. Staff Scientist
Research Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Purified the NLEE protein performed methylation assays.

Name:	Ayyanathan, Kasirajan
Project role:	Sr. Staff Scientist
Research Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Performed critical subcloning and mutagenesis.

Name:	Morgan Bailey
Project role:	Research Assistant
Research Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Supported all above for routine laboratory duties.

Name:	Elena Clancy
Project role:	Summer Lab Assistant
Research Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2
Contribution to Project:	Supported all above for routine lab duties.

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: None

9. APPENDICES:

Journal article:
Yao Q, Zhang L, Wan X, Chen J, Hu L, Ding X, Li L, Karar J, Peng H, Chen S, Huang N, Rauscher FJ 3rd, Shao F. Structure and specificity of the bacterial cysteine methyltransferase effector NleE suggests a novel substrate in human DNA repair pathway. PLoS Pathog. 2014 Nov 20;10(11):e1004522. doi: 10.1371/journal.ppat.1004522. eCollection 2014 Nov. PubMed PMID: 25412445; PubMed Central PMCID: PMC4239114.

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4. Weston, R., H. Peeters, and D. Ahel, *ZRANB3 is a structure-specific ATP-dependent endonuclease involved in replication stress response.* Genes Dev, 2012. **26**(14): p. 1558-72.
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11. Jin, J., et al., *Epigenetic regulation of the expression of Il12 and Il23 and autoimmune inflammation by the deubiquitinase Trabid.* Nat Immunol, 2016. **17**(3): p. 259-68.