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Characterizing the NF-κB pathway of the innate immune system in the sea anemone Exaiptasia pallida

Maria V. Ingersoll , '20

Characterizing the NF-κB pathway of the innate immune system in the sea anemone *Exaiptasia pallida*

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Abstract

Coral reefs are unique and globally important ecosystems that provide vital services to marine life as well as to human health and the economy. The survival of the reef ecosystem is based upon an endosymbiotic relationship between the coral and a photosynthetic microalgae of the family Symbiodiniaceae that inhabit the coral cells. The present study incorporates the use of a model organism for the Symbiodiniaceae-cnidarian symbiosis - *Exaiptasia pallida* - and cell biology methodology to characterize a specific pathway of the immune system hypothesized to play a key role in symbiosis: the NF-κB pathway. I identify three proteins, Aiptasia IκBcl3-A, IκBcl3-B, and IκBcl3-C, as homologs for either IκB or Bcl3 proteins within the Aiptasia NF-κB pathway. Through sequence and conserved domain analysis as well as characterization of protein-protein interactions, I propose a novel pathway of NF-κB induction in which IκBcl3-A may act as an IκBα homolog in complex with IκBcl3-C, as well as a Bcl3 homolog along with IκBcl3-B. The molecular characterization presented in this study provides targets for genetic manipulations aimed at studying the regulatory roles that particular proteins play in cnidarian symbiosis and immunity.

Introduction

Coral reefs are one of the most biodiverse as well as one of the most vulnerable ecosystems on the planet. Not only are they important for the plethora of unique species that rely on the reefs for protection, habitat, and food, coral reefs are important to human populations as well (Moberg and Folke, 1999). They provide protection for coastal communities from natural disasters, and commercial fishing relies heavily on species that reside around reefs (Moberg and Folke, 1999). The trophic basis of the reef ecosystem depends on endosymbiotic dinoflagellates of the family Symbiodiniaceae (previously classified as the genus *Symbiodinium*; LaJeunesse et al., 2018) which are intracellular inhabitants of the coral cells and provide their host with fixed carbon (sugar) from photosynthesis. In exchange, the corals give the Symbiodiniaceae shelter and nitrogen (reviewed by Apprill, 2020). However, this intimate relationship is threatened by changing ocean conditions. Anthropogenic changes like increased ocean temperature and acidification (due to the formation of carbonic acid from increasing levels of dissolved CO_2) lead

to a phenomenon known as bleaching, in which the intracellular microalgae are eliminated or are lost from the cnidarian host cells. If conditions do not return to normal and Symbiodiniaceae do not repopulate the host cells, the coral will eventually die.

The exact mechanisms of symbiont uptake, maintenance, loss, and repopulation remain to be completely understood. In order to ameliorate the increase in coral fatality observed over the past century, it is important to understand not only the external pressures that lead to coral bleaching and death, but the physiological, cellular, and molecular responses of both symbiotic partners to these abiotic stressors. In a review of the cellular mechanisms of bleaching published in 2008, Virginia Weis discusses the increase in reactive oxidative species (ROS) in both the symbiont and the host caused by thermal and UV stress which lead to membrane damage, a host immune response, and eventually bleaching. Further studies examine the innate genetic and proteomic variations between different Symbiodiniaceae and their cnidarian hosts that contribute to differences in bleaching resilience and reactivity to stress (reviewed by Apprill, 2020; reviewed by Tomanek, 2011). Finally, still more studies investigate the key regulatory role the host immune system plays in the symbiotic relationship and in response to environmental perturbations (reviewed by Mansfield and Gilmore, 2019). However, a narrative connecting environmental stress to the cellular response that leads to cnidarian bleaching remains incomplete.

To facilitate research into the cellular and molecular mechanisms that regulate the Symbiodiniaceae-cnidarian symbiosis, the sea anemone *Exaiptasia pallida* (here-on referred to as Aiptasia) has been established as a key model organism. Aiptasia harbor symbionts of the same clades of Symbiodiniaceae as coral, but, in comparison to coral, Aiptasia can live in a

completely aposymbiotic state (cleared of all Symbiodiniaceae) for months to years (Oakley et al., 2016; reviewed by Weis et al., 2008). In addition, Aiptasia are hardy, can be biochemically manipulated, grow relatively quickly, can reproduce asexually, and have been made to spawn in laboratories (Grawunder et al., 2015; reviewed by Weis et al., 2008). Larval (naive to Symbiodiniaceae) and aposymbiotic Aiptasia may be infected/reinfected with dinoflagellates in the laboratory (Bucher et al., 2016; reviewed by Weis et al., 2008). Finally, and of key importance to this study, as of 2015, Aiptasia has an annotated genome (Baumgarten et al., 2015).

The following study focuses on one aspect of the cellular approach to studying the Symbiodiniaceae-cnidarian symbiosis: the immune system, specifically as it functions in the model organism Aiptasia. While vertebrates have two forms of immunity – the adaptive and the innate immune systems – invertebrates, such as cnidarians, only have the innate immune system (reviewed by Mansfield and Gilmore, 2019). The innate immune system is, therefore, the manner by which coral and anemones must discern a perceived threat from the multitude of "non-self" signals to which they are constantly exposed in their marine environment. It also must be involved in uptake and maintenance of the obligate Symbiodiniaceae, whether this is by the microalgae evading detection by the immune system, downregulating the immune system during symbiosis establishment, or by some other mechanism (reviewed by Weis et al., 2008).

A key pathway within the innate immune system is the Nuclear Factor-κB (NF-κB) pathway. Induction of the NF-κB pathway – which culminates in the activation of a member of the subfamily of transcription factors called NF-κB proteins– results in the expression of proteins involved in the inflammatory response, such as cytokines and chemokines, as well as proteins

involved in host-pathogen interactions such as Nitric Oxide Synthase (reviewed by Weis, 2008). The NF-κB pathway is highly conserved and has been at least partially annotated in species from corals to fruit flies to humans (reviewed by Gilmore, 2006). In higher order taxa such as humans, there are two well-characterized NF-κB pathways: the canonical and the non-canonical pathways (Figure 1). These two pathways differ in the signals recognized by the cell surface receptors and in the signaling proteins that lead to NF-κB activation; they also differ in the specific NF-κB family member that is ultimately activated (reviewed by Gilmore, 2006 and Sun, 2017).

The canonical pathway (Figure 1A) is initiated via binding of a ligand to immune receptors in the membrane such as Pattern Recognition Receptors and cytokine receptors. The intracellular domain of the receptor induces proteins that activate a complex of IκB kinase molecules (IKKα and IKKβ), bound by the scaffold protein NEMO (also called IKKγ), via phosphorylation on IKKβ. The activated IKK complex may then in turn phosphorylate the N-terminus of a member of the Inhibitor of κB family (in the canonical pathway, this is IκBα). When the canonical pathway is inactive, IκBα is in complex with members of the NF-κB protein family, such as p105 and RelA or c-Rel. When phosphorylated by the IKK complex, IκBα is targeted for degradation through polyubiquitination, and p105 is processed to p50, which has an exposed nuclear localization signal. p50, heterodimerized with RelA or c-Rel, is now free to enter the nucleus and aid in transcription of immune response genes (reviewed by Gilmore, 2006 and Sun, 2017).

The non-canonical pathway (Figure 1B) involves initiation by Tumor Necrosis Factor Receptors in the membrane, the intracellular domain of which activates the NF-κB Inducing

Kinase (NIK), which then phosphorylates and activates IKKα. IKKα phosphorylates select serine residues near the carboxy-terminal of the p100 NF-κB family member. This targets p100 processing to p52 which can then translocate to the nucleus and bind to RelB, forming a transcriptional activator (reviewed by Gilmore, 2006 and Sun, 2017).

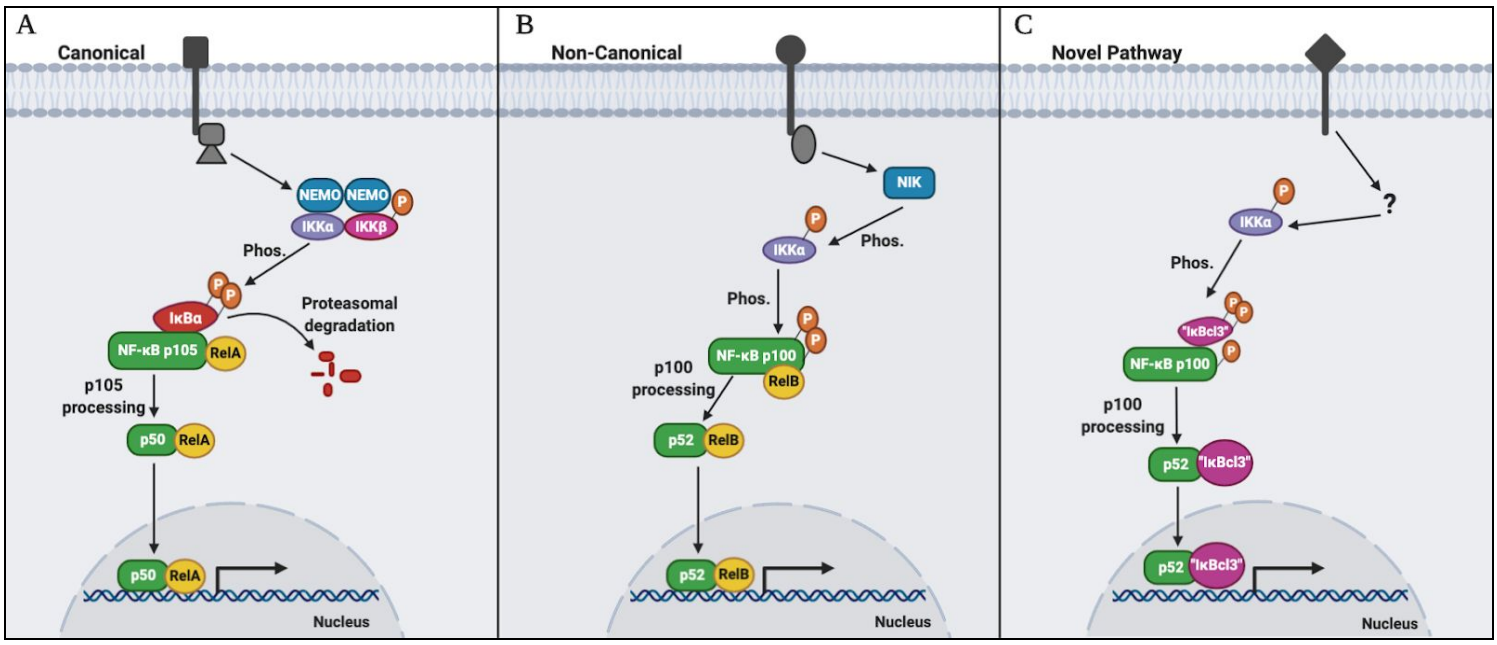


Figure 1. Induction of distinct NF-κB pathways. (**A)** The canonical pathway is present in higher order taxa and is induced by ligand binding to cell surface immune receptors. The IKK complex is activated and in turn phosphorylates IκBα. IκBα is then polyubiquitinated and degraded, freeing NF-κB p105, in complex with RelA, to be processed to its active form: p50. The p50:RelA complex can now enter the nucleus and induce transcription of immune response genes. **(B)** The non-canonical pathway is also present in higher order taxa and is induced by ligand binding to cell surface immune receptors distinct from those in the canonical pathway. IKKα is activated and directly phosphorylates NF-κB p100 (dimerized to RelB). Phosphorylated p100 is processed to the active p52. The p52:RelB complex can now enter the nucleus and induce transcription of distinct immune response genes. **(C)** A novel NF-κB pathway is hypothesized to take the place of the canonical and non-canonical pathways in Aiptasia. This pathway is reminiscent of the non-canonical pathway in that it induces p100 processing to p52. However, this pathway lacks any Rel protein homolog and may be regulated by one or more Aiptasia IκBcl3 proteins that function as IκBα or Bcl3 homologs. .

Importantly, many proteins within both NF-κB pathways contain regions of high homology with other members of the pathways. For example, the C-terminal regions of NF-κB p100 and p105 contain domains that closely resemble and perform similar functions to IκBα, i.e.,

inhibiting transcriptional activation. The C-termini of NF-κB p100 and p105 and the IκB superfamily proteins contain 4-7 ankyrin repeats within their Ankyrin repeat domain (ANK). Ankyrin repeats are sequence motifs that mediate protein-protein interactions (Li et al., 2006). This region is removed during p100 and p105 processing to form p52 and p50, respectively (reviewed in Huxford and Ghosh, 2009 and by Sun, 2017;). Additionally, within the IκB super-family are the Bcl3 proteins. Bcl3 proteins perform distinct functions to classic members of the IκB subfamily, such as IκBα, though they are structurally very similar; specifically, Bcl3 proteins aid in transcriptional induction by dimerizing with p50 and p52 proteins in the nucleus (reviewed by Hatada et al., 1999 and by Huxford and Ghosh, 2009). Bcl3 proteins also require specific phosphorylation by a number of kinases, which include IKKα/β, in order to be active, and Bcl3 proteins undergo polyubiquitination-dependent degradation, yet this protein turnover is regulated distinctly from the turnover of IκBα. It should be noted, however, that IKKα/β phosphorylation of Bcl3 proteins occurs at the C-terminus, as opposed to the N-terminus of IκBα (Wang et al., 2017; reviewed by Zhang et al., 2017).

The NF-κB pathway has been hypothesized to play a key role in cnidarian bleaching, as it has been reported to be downregulated in Aiptasia during active symbiosis with Symbiodiniaceae and upregulated following loss of symbionts (Mansfield et al., 2017). The Aiptasia genome is annotated as containing only one member of the NF-κB protein superfamily: a homolog to the mammalian p100 protein (Baumgarten et al., 2015; Mansfield et al., 2017; Williams et al., 2018). The Aiptasia NF-κB protein contains a putative site of IKKα phosphorylation in the C-terminus (Mansfield et al., 2017).

It is unlikely that Aiptasia uses either a standard canonical or non-canonical NF-κB signaling pathway. There is currently no direct evidence that the Aiptasia NF-κB p100 is inhibited by an IκBα homolog when the pathway is inactive. The ambiguity of a known inhibitor of NF-κB and the observation that the p100 homolog may function as its own inhibitor suggest that Aiptasia utilizes a non-canonical NF-κB pathway, under which phosphorylation and cleavage of the C-terminus of NF-κB is necessary and sufficient for transcriptional activation. However, the Aiptasia genome contains three proteins within the IκB superfamily (which includes the IκB and Bcl3 subfamilies) whose roles are unknown (Baumgarten et al., 2015). I will refer to these three proteins as IκBcl3 proteins. Additionally, a NIK homolog is absent from the Aiptasia genome, which is needed for activation of IKKα in the non-canonical pathway. Finally, in other systems, dimerization of the processed form of NF-κB - p50 or p52 - with other members of the NF-κB superfamily - RelA and RelB respectively - must occur in order for transcription of target immune response genes to be activated in both the canonical and non-canonical pathways. p50 and p52 can bind to the target gene sequence via their Rel-Homology Domains (RHD), but the transactivation domains (TAD) of RelA and RelB are required to initiate transcription (reviewed by Huxford and Ghosh, 2009). Aiptasia does not contain any homologs of the Rel subfamily (the other subfamily within the NF-κB protein superfamily), which includes RelA, c-Rel, and RelB (reviewed by Gilmore, 2006). Together, these data suggest that the Aiptasia NF-κB pathway may be under a novel mechanism of control (Figure 1C) (Baumgarten et al., 2015; Mansfield et al., 2017; Williams et al., 2018).

Here I utilize the Yeast-Two Hybrid (YTH) assay as a method of characterizing the unique pathway of NF-κB activation in Aiptasia by analyzing the interactions between Aiptasia

proteins believed to play a key role in NF-κB regulation. YTH is a technique that utilizes the Gal4p transcription factor and reporter genes in the yeast *Saccharomyces cerevisiae* in order to test interactions between proteins of interest. Gal4p has an activation domain and a DNA-binding domain. The sequence encoding a domain from Gal4p is fused to the sequence of an Aiptasia gene to be assayed, and plasmids containing these chimeric genes are transformed into yeast. If the two Aiptasia proteins interact, the Gal4p transcriptional activation and DNA binding domains are brought into proximity and reporter genes are transcribed, allowing for a visual assessment of protein-protein interactions (Matchmaker Gold Yeast Two-Hybrid System User Manual).

I hypothesize that the Aiptasia NF-κB pathway operates under a previously uncharacterized mechanism of control that will be key for future studies that attempt to understand the role NF-κB plays in Symbiodiniaceae-cnidarian symbiosis and bleaching. I specifically hypothesize that three Aiptasia proteins within the IκB superfamily (IκBcl3 proteins) may be further characterized as IκBα-like proteins or Bcl3-like proteins, which I will determine by analyzing peptide sequence homology to known IκB subfamily proteins and Bcl3 proteins, as well as by analyzing protein binding partners in Yeast Two Hybrid.

Materials and Methods

Sequence Homology

Gene selection

Translated cDNA sequences from *Nematostella vectensis* (Nv) (Brennan et al., 2017) for IKKα, IκBα, and NF-κB were used as query sequences in BLASTp searches against the Aiptasia proteome (reefgenomics.org). Results of the search are presented in Table 1.

Table 1. Parameters for Aiptasia gene identification, amplification, and sequencing. PCR product sizes are measured as base pairs and include recombinant sequences. Primer annealing temp is for PCR reactions and is in ℃.
* = primers used only with inserts in pGBKT7; ** = primers used only with inserts in pGADT7.

Aiptasia Gene Name	Aiptasia protein homolog	*N. vectensis* protein homolog	Aipatsia PCR product size	PCR primers	Primer annealing temp	Sequencing Primers used
Aipgene8842	IKKα	IKKα	1493	n/a	n/a	T7 (GENEWIZ)
Aipgene8848 + Aipgene8884	NFκB full-length (p100)	NFκB	2576	n/a	n/a	T7 (GENEWIZ) NFκB Internal Forward: 5' CTCAAATCCCGTCTACGACAG 3' NFκB Internal Reverse: 5' TAAGTCAGGCTATCGACATCGCAC 3' pGBKT7 Reverse*: 5' CCTCAAGACCCGTTTAGAGGCC 3' pGADT7 Reverse**: 5' CAGTATCTACGATTCATCTGCAGCTCGAGCTCG 3'
Δ427	p52	NFκB	1298	Forward: 5' **CATGGAGGCCGAATTC**ATGACACACAG 3' Reverse: 5' **GCAGGTCGACGGATCC**ATCAAAAAGAAA CCCGGACCCAAAATTGAAC 3'	57.5	T7 (GENEWIZ) NFκB Internal Forward: 5' CTCAAATCCCGTCTACGACAG 3' pGBKT7 Reverse*: 5' CCTCAAGACCCGTTTAGAGGCC 3' pGADT7 Reverse**: 5' CAGTATCTACGATTCATCTGCAGCTCGAGCTCG 3'
Aipgene18872	IκBcl3-A	IκBα	1163	Forward: 5' **CATGGAGGCCGAATTC**ATGGATAAGGAAA GGAAACTGACTG 3' Reverse: 5' **GCAGGTCGACGGATCC**TTACAGCAGAGTT TTCAGCGTATTTAG 3	54	T7 (GENEWIZ)
Aipgene25081	IκBcl3-B	IκBα	1184	Forward: 5' **CATGGAGGCCGAATTC**ATGATTTTGTTGGA TTTAGTGCCGTCC 3' Reverse 5' **GCAGGTCGACGGATCC**TTATGATT TTGACCTCTTAGAGGTAGACTGTTCAG 3'	57.5	T7 (GENEWIZ)
Aipgene25087	IκBcl3-C	IκBα	1376	Forward: 5' **CATGGAGGCCGAATTC**ATGGTAGCATCAAGC AGTCAGGAAAACTCGA 3' Reverse: 5' **GCAGGTCGACGGATCC**TGTGAACATGTCA GAAAATTGACATATCTCATATCATCATGTC 3'	57.5	T7 (GENEWIZ)
	-recombinant sequences are bolded					

Homology Comparisons

Preliminary relationships of homology between Aiptasia IκBcl3 proteins and homologous Nv and human proteins were taken from the closest hits in the Aiptasia proteome in a BLASTp analysis against a Nv or human query (Table 2). Conserved domain analyses (Figure 3) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) were performed on the following protein homologs: Human IKKα (O15111), Nv-IKKα, Aiptasia IKKα (Aipgene8842), Human NF-κB p105 (NF-κB1; P19838), Human NF-κB p100 (NF-κB2; Q00653), Human RelA (p65; Q04206), Nv NF-κB, Aiptasia NF-κB, Aiptasia Δ427, Human IκBα (P25963), Human IκBβ

(Q15653), Human IκBε (O00221), Human Bcl3 (P20749), Nv IκB, Nv Bcl3, Aiptasia IκBcl3-A, Aiptasia IκBcl3-B, and Aiptasia IκBcl3-C (Aipgenes 18872, 25081, and 25087 respectively) (human amino acid sequences were obtained from UniProt FASTA; Nv sequences were obtained from Brennan et al., 2017; Aiptasia NF-κB sequences were obtained from Williams et al., 2018, and all other Aiptasia sequences were obtained from Baumgarten et al., 2015).

Previous studies of human IκBα have determined that serines 32 and 36 of IκBα must be phosphorylated by the IKKα/β complex in order for IκBα to be tagged for degradation and for NF-κB p105 to be processed (and therefore activated) (Huxford and Ghosh, 2009; Traenckner et al., 1995; reviewed by Zhang et al., 2017). Manual sequence alignments of the human IκBα peptide sequence to those of the three Aiptasia IκBcl3s were performed in order to determine likely residues of equivalent function to S32 and S36 of human IκBα.

Finally, PEST domains (regions rich in proline, glutamic acid, serine, and threonine) of Aiptasia IκBcl3s and Aiptasia NF-κB were predicted by two methods. In the first method, concentrations of each PEST residue were measured as a percent of the total amino acids in the C-terminus (after the most C-terminal ankyrin repeat) of each peptide sequence. This percentage was compared to the percentage of the same residue in the rest of the peptide. This comparison was performed in order to qualitate enrichment of proline, glutamic acid, serine, and threonine. In the second method, each peptide sequence was run through the EMBOSS epestfind algorithm (http://bioinfo.nhri.org.tw/cgi-bin/emboss/epestfind) verified by Qile and colleagues in 2019.

Yeast Two Hybrid

Plasmids

The fusion constructs were made by cloning the genes encoding the proteins of interest into distinct plasmids (Figure 2): the plasmid pGADT7 (GAD) encodes the Gal4p activation domain, as well as a gene encoding resistance to ampicillin (Amp^R) for selection in *E. coli* and the *LEU2* gene for selection in yeast; the plasmid pGBKT7 (GBK) encodes the Gal4p DNA-binding domain, as well as a gene encoding resistance to kanamycin (Kan^R) for selection in *E. coli* and the *TRP1* gene for selection in yeast (Matchmaker Gold Yeast Two-Hybrid System User Manual).

Cloning genes of interest

The Aiptasia gene annotated as that encoding NF-κB - Aipgene8848 - only contained the sequence for the N-terminal domain of full-length NF-κB, while a second gene - Aipgene8884 - encoded the sequence for the C-terminal domain. It had been previously suggested (Williams et al., 2018) that this was a sequencing artifact. The sequence containing the entire predicted NF-κB coding sequence, along with Aipgene8842 (encoding IKKα), were yeast-codon optimized, and EcoRI and BamHI restriction enzyme sites were removed. Regions of homology necessary for cloning by recombination were added, and the two genes were synthesized by GENEWIZ.

Aipgenes 18872, 25081, and 25087 (encoding the three Aiptasia IκBcl3s) were amplified by PCR (New England BioLabs Phusion Hot Start Flex DNA Polymerase) from cDNA. Primers and annealing temperatures are listed in Table 1. p52 (Δ427) - the truncated form of NF-κB (Mansfield et al., 2017) - was amplified from the full-length NF-κB gene using New England

BioLabs Phusion Hot Start Flex DNA Polymerase kit (Table 1). Gel electrophoresis of the PCR products was performed to confirm successful amplification, and Nucleospin columns were utilized to purify products before cloning. Takara Bio In-Fusion® HD Cloning Kit was used to clone Aiptasia genes into both GAD and GBK vectors. The In-Fusion reactions were transformed into Stellar Competent *Escherichia coli* cells and plated onto media containing the appropriate antibiotic to each plasmid (Takara Bio Clontech Matchmaker® Gold Yeast-Two Hybrid User Manual). The Qiagen QIAprep Spin Miniprep Kit was used to isolate DNA from *E. coli* transformants. In order to assure that an insert of the proper size was contained in each plasmid (Table 1 for expected bp size of each Aiptasia gene insert), DNA was digested with EcoRI and BamHI restriction enzymes. Plasmids with inserts of the expected size were sequenced (GENEWIZ). All plasmids were sequenced with the T7 primer (GENEWIZ). In addition, the NF-κB- and Δ427-containing plasmids were sequenced with additional primers not provided by GENEWIZ (included in Table 1). Sequences containing the fewest mutations when compared to the canonical database sequence were used for subsequent experiments. In the cases in which all sequences contained mutations, replicates were used (i.e. GBK-IκBcl3-B.1 and GBK-IκBcl3-B.2) to analyze the possible role of the mutations to the protein function.

Transformation into yeast

Plasmids purified by miniprep were transformed into yeast following the protocol outlined in the Takara Bio Yeastmaker Yeast Transformation System 2 for a small scale batch. GAD vectors were transformed into haploid Y187 cells, and GBK vectors were transformed into haploid Y2HGold cells (Takara Bio Clontech Matchmaker® Gold Yeast-Two Hybrid User Manual).

Mating and selection for diploids

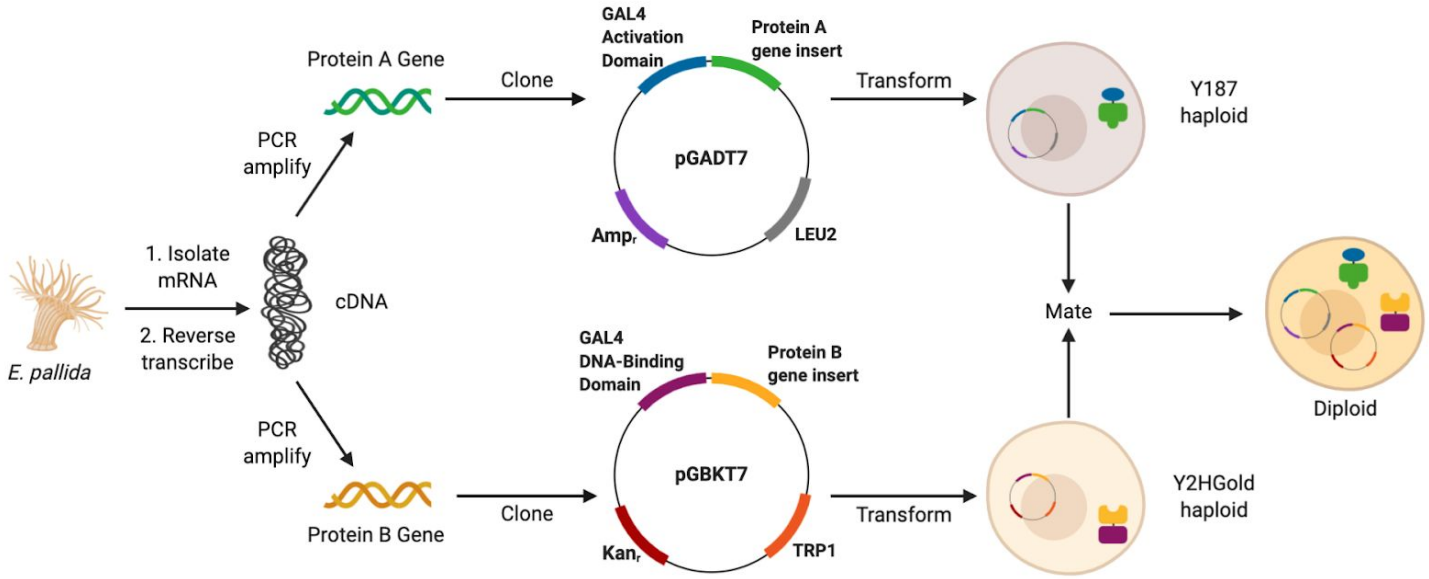
Y187 or Y2HGold transformants were patched onto selective plates. Transformants of the other strain were grown to saturation in selective liquid media and spread and grown on YPD plates. After mating was initiated by replica-plating, diploids containing both plasmids were selected on plates lacking both Leucine and Tryptophan (Double Drop-Out, or DDO) (Takara Bio Clontech Matchmaker® Gold Yeast-Two Hybrid User Manual).

Screen for interactors

Diploids grown on DDO plates were patched onto three plates of different selective media conditions: DDO, Quadruple Drop-Out (QDO), and DDO X-α-Gal/Aureobasidin A (DDO X/A) (Takara Bio Clontech Matchmaker® Gold Yeast-Two Hybrid User Manual).

Quantitative testing

Positively interacting diploid cells were picked from the DDO plates and inoculated into DDO liquid media to grow to saturation. A dilution series for each positive interaction was created in a 96-well plate in sterile, deionized H_2O. Each dilution series was immediately plated onto DDO, QDO, and DDO X/A plates.



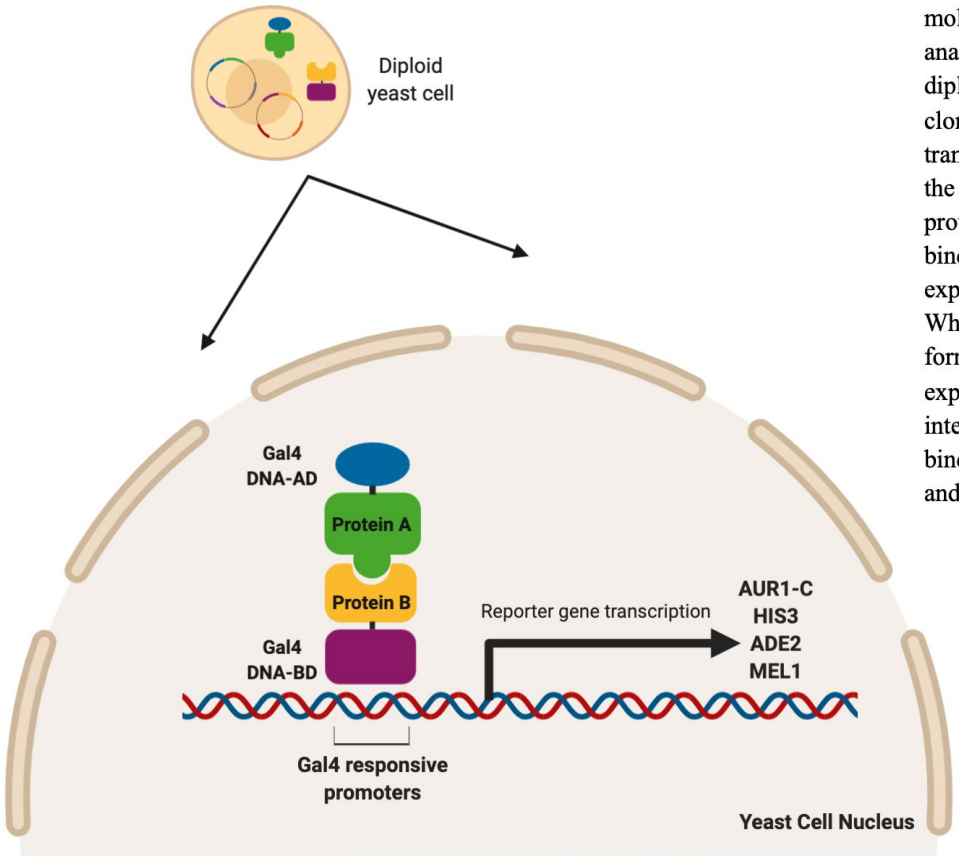


Figure 2. Yeast Two Hybrid (YTH) is a molecular technique that allows researchers to analyze protein-protein interactions within diploid yeast cells. One protein of interest is cloned to the activation domain of the Gal4p transcription factor (in pGADT7, expressed in the haploid Y187 cell line), and the other protein of interest is cloned to the DNA-binding domain of Gal4p (in pGBKT7, expressed in the haploid Y2HGold cell line). When the two haploid strains are mated to form diploids, the two proteins of interest are expressed within the same cell. If they interact, the Gal4p activation and DNA-binding domains are brought into proximity and reporter genes are expressed.

Western Blots

Each haploid transformant used in qualitative and quantitative Yeast Two Hybrid analysis was assayed for protein expression levels by SDS-PAGE and Western Blot techniques (protocol adapted from GE Healthcare Western Blotting methods). Membranes containing proteins from GAD/Y187 strains were stained with mouse-anti-HA primary antibody and goat-anti-mouse-HRP secondary antibody; membranes containing proteins from GBK/Y2HGold strains were stained with mouse-anti-MYC primary antibody and goat-anti-mouse-HRP secondary antibody. Mouse-anti-tubulin primary antibody (and goat-anti-mouse-HRP secondary antibody) was used as a loading control. Membranes were visualized utilizing ECL reagents.

Results

Analysis of sequence and structure of Aiptasia Bcl3/IκB homologs

To determine whether each Aiptasia IκBcl3 protein shared more homology with Bcl3 or IκB proteins from other systems, homology comparisons of Aiptasia IκBcl3 protein sequences were performed against human and Nv Bcl3 and IκB peptide sequences (Table 2). BLASTp analysis suggests that AipIκBcl3-C may be most likely to function as Bcl3, while AipIκBcl3-A and AipIκBcl3-B may be more likely to function as proteins belonging to the IκB subfamily.

Table 2. BLASTp results of amino acid sequence homology of Aiptasia IκBcl3 proteins to *Nematostella vectensis* (Nv) and human Bcl3 and IκB amino acid sequences

Homologous gene	Aip gene with most homology		Aip gene with least homology
Nv Bcl3	IκBcl3C	IκBcl3B	IκBcl3A
Nv IκB	IκBcl3A	IκBcl3B	IκBcl3C
Human Bcl3	IκBcl3C	IκBcl3B	IκBcl3A
Human IκBα	IκBcl3B	IκBcl3C	IκBcl3A
Human IκBβ	IκBcl3B	IκBcl3C	IκBcl3A
Human IκBε	IκBcl3A	IκBcl3B	IκBcl3C

To explore further the role each Aiptasia protein might play in the Aiptasia NFκB pathway, analyses of conserved domains and PEST regions of Aiptasia IKKα, NF-κB, Δ427, and IκBcl3 homologs were performed and compared to human and Nv homologs (Figure 3 and Table 3). In agreement with previous studies (Mansfield et al., 2017), the annotated domains in the Aiptasia NF-κB protein appear to most resemble those of human NF-κB p100, as both proteins have six Ankyrin repeats, whereas human NF-κB p105 has only four. There is no evidence of a complete RelA or RelB homolog in the Aiptasia genome, and there is no evidence of a protein with high homology to the C-terminal transactivation domain (TAD) of the human RelA or RelB peptides. In contrast to the results from the BLASTp analysis, comparison of the overall structure and conserved domains between the Aiptasia IκBcl3 proteins and the human and Nv Bcl3 and IκB indicate that AipIκBcl3-A is most similar to Bcl3, rather than a classical IκB; AipIκBcl3-A contains six ankyrin repeats like human Bcl3. The Nv Bcl3 homolog contains only four ankyrin repeats. AipIκBcl3-B and AipIκBcl3-C, on the other hand, both contain five ankyrin repeats, comparable to the five ankyrin repeats in the human and Nv IκB proteins (Figure 3).

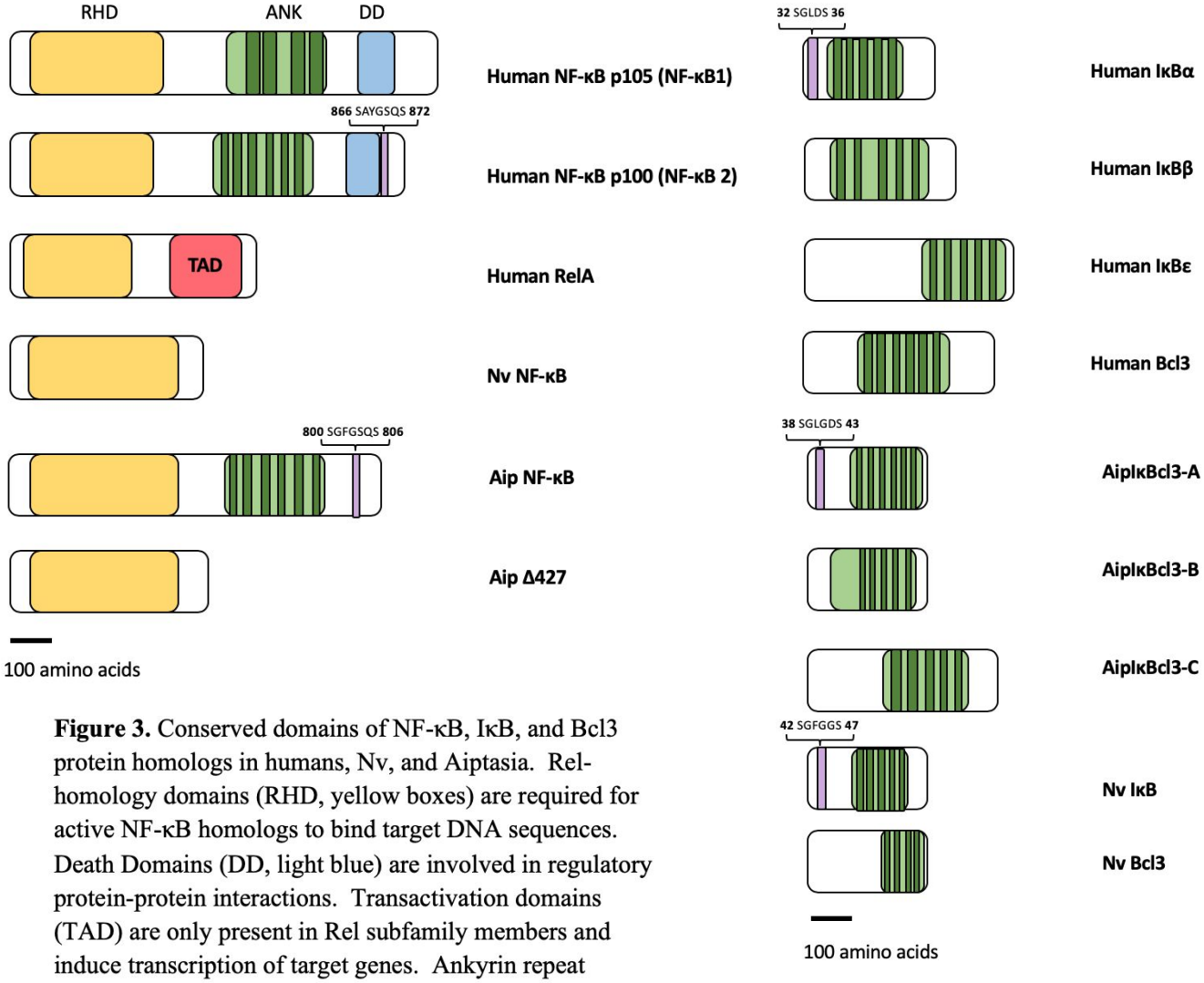


Figure 3. Conserved domains of NF-κB, IκB, and Bcl3 protein homologs in humans, Nv, and Aiptasia. Rel-homology domains (RHD, yellow boxes) are required for active NF-κB homologs to bind target DNA sequences. Death Domains (DD, light blue) are involved in regulatory protein-protein interactions. Transactivation domains (TAD) are only present in Rel subfamily members and induce transcription of target genes. Ankyrin repeat domains (ANK) are light green boxes containing dark green ankyrin repeats. Purple boxes are sites of IKK phosphorylation; target sequences are given (for the Aiptasia and Nv proteins, these sequences are putative). All proteins and domains are to scale. Scale bar is 100 amino acids.

Analyses of possible phosphorylation sites by IKKα/β within the N-termini of human IκBα, the Aiptasia IκBcl3s, and Nv IκB as well as within the C-termini of human NF-κB p100 and Aiptasia NF-κB are illustrated in Figure 3. All three Aiptasia IκBcl3 homologs had regions relatively rich in serine and threonine (able to be phosphorylated by serine-threonine kinases

such as IKKα/β). However, close analysis of the C-terminal serine residues reveals that AipIκBcl3-A presents the closest match to human IκBα. The human IκBα serines that must be phosphorylated for IκBα to be tagged for degradation are S32 and S36, which are separated by G33, L34, and D35. Human S32 and S36 may map to AipIκBcl3-A S38 and S43 (reviewed by Hayden and Ghosh, 2008, Huxford and Ghosh, 2009, and Zhang et al., 2017; Traenckner et al., 1995). These two serine residues are separated by G39, L40, G41, and D42. The only difference between the intervening residues in human IκB and AipIκBcl3-A is the extra glycine in the latter sequence. Glycine is small, nonpolar and uncharged and may only minimally change the three-dimensional structure of the protein (Figure 3).

Finally, analyses of the C-termini of each Aiptasia IκBcl3 protein, as well as Aiptasia NF-κB, were performed in search of PEST domains (regions rich in proline, glutamic acid, serine, and threonine), which are common to proteins that undergo rapid turnover and are characteristically defined as being present in IκB but not Bcl3 homologs (reviewed by Huxford and Ghosh, 2009 and Zhang et al., 2017). PEST concentrations in Aiptasia proteins C-terminal to the final ankyrin repeat were compared to PEST concentrations throughout the rest of the protein. PEST concentrations in the C-termini of all proteins were compared to PEST concentrations in human IκBα and human Bcl3. Table 3 presents the results from these analyses as percentages. From this assessment, it was concluded that the C-terminal of AipIκBcl3-A most resembles that of human IκBα, followed by the C-termini of AipIκBcl3-B, and finally by AipIκBcl3-C. PEST scores for each sequence were also determined using a previously validated algorithm (Qile et al., 2019), however none of the three Aiptasia IκBcl3s yielded scores sufficient to be considered potential PEST motifs.

Table 3. Determination of C-terminal PEST domains in human, Nv, and Aiptasia IκB and Bcl3 protein homologs. C-terminal includes amino acids following the final ankyrin repeat. N-terminal is defined as preceding this point. Based on the percent PEST in the C-termini and N-termini, the gradient indicates proteins that are most similar. Emboss PEST score was determined using a previously-validated algorithm by which a PEST score of above 5.0 was considered a potential PEST domain. A score of lower than 5.0, including negative numbers, was considered a poor PEST domain. (--) indicates that no PEST domain was identified in the C-terminus. *indicates that a potential PEST domain was identified in another location within the protein.

Protein Homolog	Percent PEST in C-term	Percent PEST in N-term	C-term Emboss PEST score
Human IκBα	38%	23%	5.13
Human IκBβ	50%	23%	12.62
Human IκBε	34%	27%	4.16*
Human Bcl3	45%	26%	13.25
Nv IκB	32%	27%	-10.83*
Nv Bcl3	13%	24.50%	-11.29
AipIκBcl3-A	36%	22%	-9.8
AipIκBcl3-B	36%	24%	--
AipIκBcl3-C	31%	27.50%	--
Aiptasia NF-κB	35%	25%	7.79

From these analyses of Aiptasia IκBcl3 protein sequence and structural homology to previously annotated IκB and Bcl3 proteins, I predicted that AipIκBcl3-A would bind to AipIKK and full-length AipNF-κB in YTH (most like an IκBα protein) and AipIκBcl3-C would bind to AipΔ427 in YTH (most like a Bcl3 protein).

Expression level of Aiptasia proteins in haploid yeast

Western blot analyses of Aiptasia proteins revealed that the levels of the fusion proteins varied when compared to the levels of tubulin used as a loading control (Figure 4).

A

Protein	NF-κB	Δ427	IKK	IκBcl3-A	IκBcl3-B	IκBcl3-C	+ ctrl
Expected MW in GBK	115 kD	70.5 kD	78 kD	63 kD	64 kD	69.5 kD	65.5 kD
Expected MW in GAD	109 kD	64.5 kD	72 kD	57 kD	58 kD	63.5 kD	97.5 kD

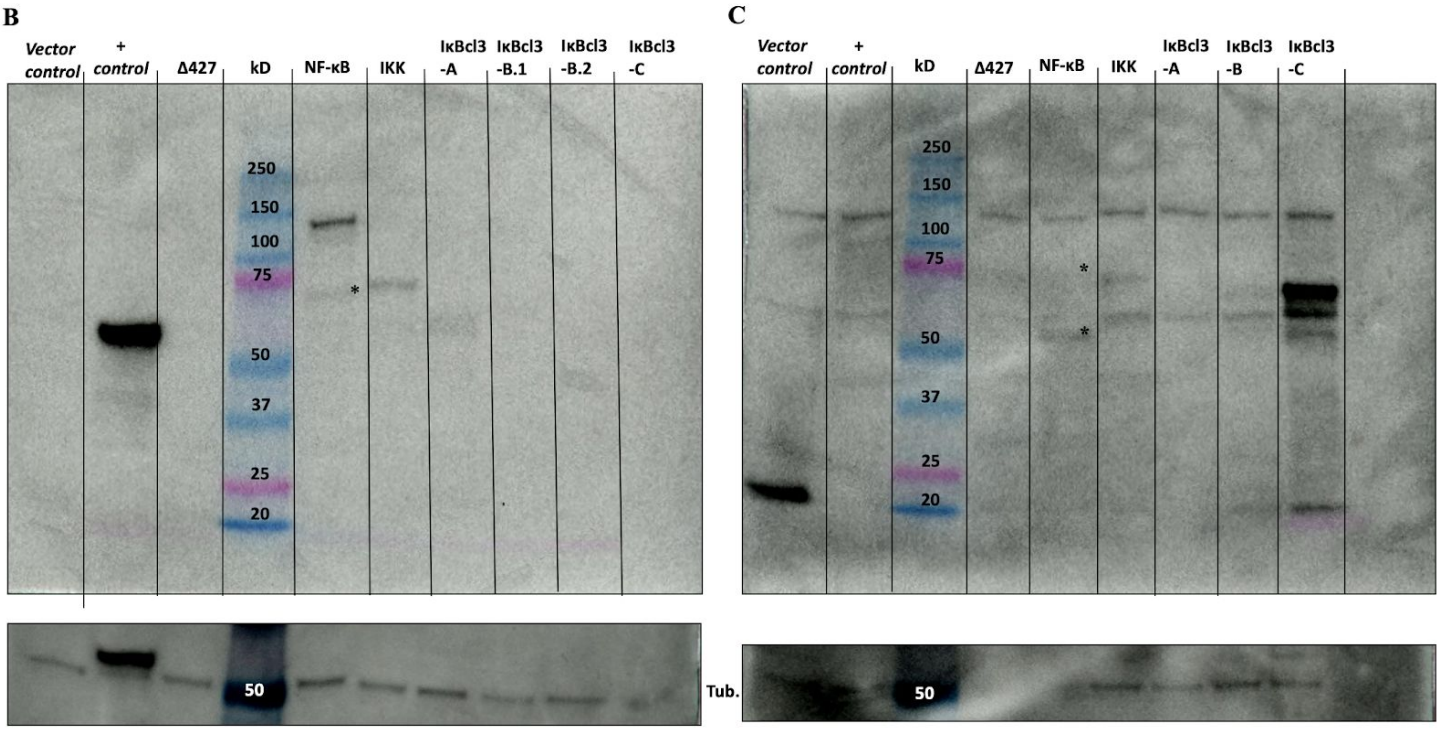


Figure 4. Aiptasia protein expression in haploid yeast as determined by Western Blot. **(A)** Expected molecular weights (in kD) of Aiptasia proteins fused to Gal4p DNA-binding domain (GBK) or Gal4p activation domain (GAD). **(B)** Upper panel: Aiptasia proteins expressed from pGBKT7. Lower panel: tubulin loading control. The dark band above the tubulin band in the second well is signal from the positive control protein. **(C)** Upper panel: Aiptasia expressed from pGADT7. Lower panel: tubulin loading control. * mark likely cleaved products of NF-κB.

While full-length AipNF-κB was observed on the blot examining pGBKT7-expressed proteins, AipNF-κB appeared on both blots at molecular weights lower than that which was expected for the full-length protein (asterisks in Figure 4B and 4C). This suggests that full-length AipNF-κB is undergoing some form of processing within the yeast cell. Based on the size of the proteins and the knowledge that the tag is at the amino terminus, it may be that this cleavage product is similar to p52/Δ427 protein, and is worth consideration when analyzing

results from Yeast Two Hybrid. Taking into account the tubulin loading control, GBK-AipNF-κB was most highly expressed, followed, in order of expression, by GBK-AipIKK, GBK-AipIκBcl3-A, and GBK-AipIκBcl3-B replicate 2 (GBK-IκBcl3-B.2). GBK-AipΔ427, GBK-AipIκBcl3-B replicate 1 (GBK-IκBcl3-B.1) had very low expression levels (evidenced by no visible bands on the blot), yet both proteins interact with other proteins in Yeast Two Hybrid (see below), indicating that some level of these proteins is being expressed. Finally, given the low levels of tubulin detected in the strain expressing GBK-AipIκBcl3-C, it is difficult to determine the relative amounts of expression of this construct (Figure 4B).

Blots of Aiptasia proteins expressed from pGADT7 plasmids appeared to contain relatively equal amounts of total protein loaded in all wells except for those of GAD-AipΔ427 and GAD-AipNF-κB, which had lower levels of tubulin. The antibodies used to stain this blot also appeared to bind to at least two unknown yeast proteins located at molecular weights of about 125 kD and 60 kD which can also be used to compare levels of protein between lanes. Taking into account the apparent concentrations of loaded protein based on the cross-reacting bands and tubulin levels, GAD-AipIκBcl3-C was most highly expressed in haploid Y187 cells, followed, in order of expression, by GAD-AipNF-κB (though there was very little total loaded protein in this well, a NFκB band was observable), GAD-AipΔ427, GAD-AipIKKα, GAD-AipIκBcl3-B, and finally GAD-AipIκBcl3-A (Figure 4C). The high concentration of GAD-AipIκBcl3-C and the low concentration of GAD-AipIκBcl3-A is consistent with the hypothesis that AipIκBcl3-A contains a PEST domain whereas AipIκBcl3-C does not. Yeast can recognize and degrade PEST sequences, inducing the rapid turnover of PEST-containing proteins, which may explain the low concentration of GAD-AipIκBcl3-A (Marchal et al., 1998).

Protein-Protein Interactions in Yeast-Two Hybrid

The Yeast-Two Hybrid assay was performed in order to characterize interactions between specific proteins hypothesized to play a role in the NF-κB pathway within Aiptasia (Figure 5). Quantitative analyses to test the strength of the interactions were performed on a subset of protein pairs (Figure 5A). Those not included in the quantitative screen were analysed by a qualitative assay only (Figure 5B). Only a subset of diploids were analyzed quantitatively because of time constraints. AipIκBcl3-A, AipΔ427, and AipNF-κB were strong autoactivators when cloned to the DNA Binding Domain transcript in pGBKT7 (able to recruit transcription machinery and induce expression of reporter genes without interacting with the DNA activation domain), as evidenced by growth and blue coloring when mated with the negative control on DDO X/A media (Figure 5C and data not shown). It was therefore impossible to test by this methodology homodimerization or interactions between any of these proteins. However, autoactivation of AipIκBcl3-A indicates that this protein may behave as a transcription factor and therefore function as a Bcl3-like protein in Aiptasia. Because AipΔ427 was also able to induce transcription of reporter genes even in the absence of the Gal4p activation domain, it is possible that, in Aiptasia, heterodimerization between a Rel protein and the processed form of NF-κB is not necessary to induce transcription of NF-κB target genes. Finally, the Westen blot of GBK-AipNF-kB provides evidence that AipNF-kB is being cleaved into a smaller protein within the yeast cell. If this cleaved product is a result of the removal of the C-terminal inhibitory domain, it could explain why AipNF-kB autoactivates in YTH: it functionally becomes p52.

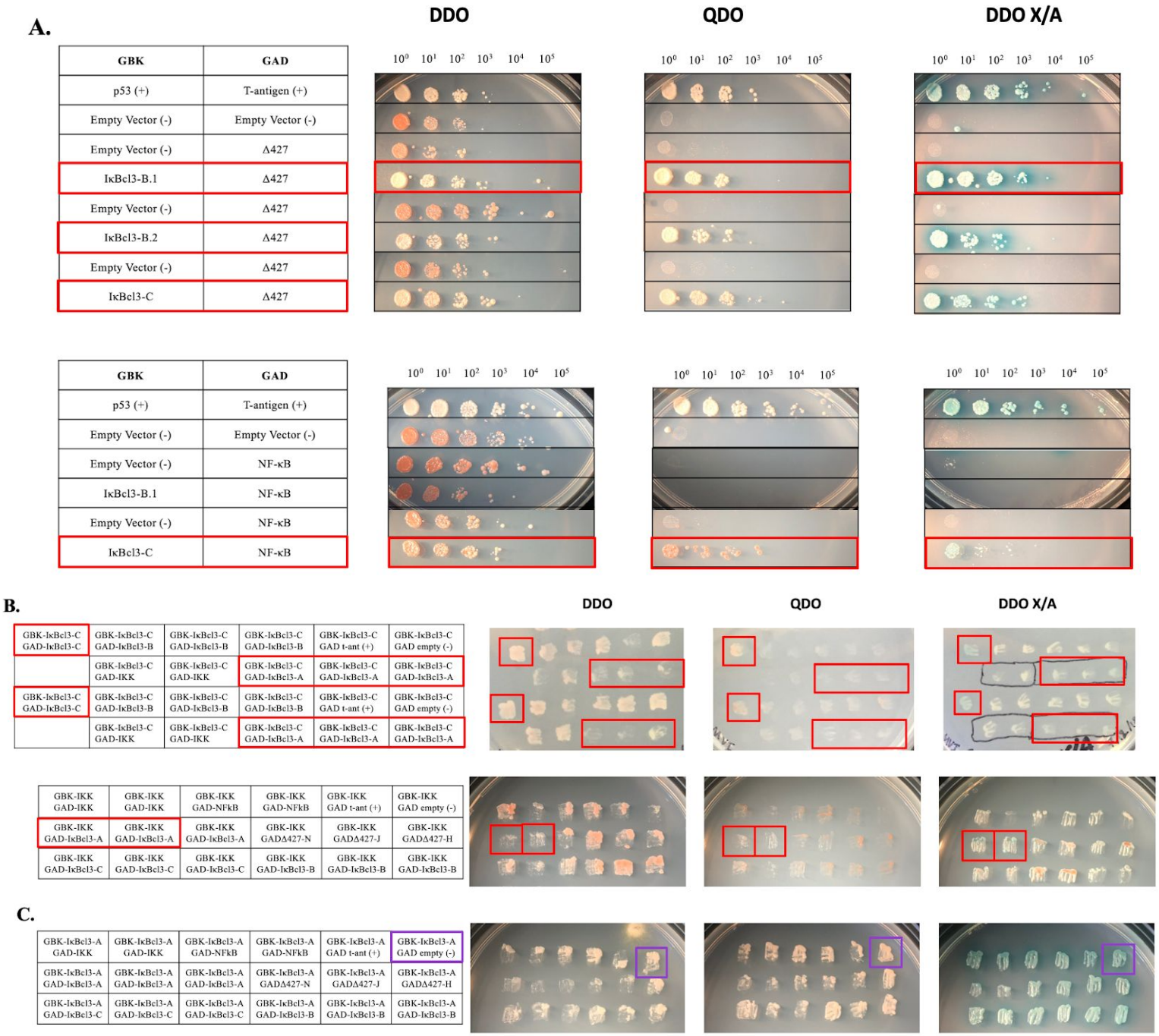


Figure 5. Yeast Two Hybrid (YTH) assay determines protein-protein interactions between Aiptasia proteins. DDO plates assured presence of both Y2H plasmids within yeast cells; QDO and DDO X/A plates tested for interactions between Aiptasia proteins and selection for presence of plasmids. Red boxes are observed interactions. Multiple transformants of each gene were analyzed, but only one replicate of each, representing an average result, is presented here. Some variability present between replicate transformants is likely because of differences in expression. **(A)** Quantitative YTH analysis. Serial dilutions performed on diploid cell culture and immediately plated. **(B)** Qualitative YTH analysis performed on all diploids. Shown here are plates with interactions not tested quantitatively. **(C)** AipIκBcl3-A is an autoactivator that can induce reporter gene expression without recruiting Gal4p activation domain (purple boxes). AipNF-κB and AipΔ427 are also autoactivators (data not shown).

Figure 6 provides a summary of all observed protein-protein interactions from quantitative and qualitative Yeast Two Hybrid screens. Relative strengths of interactions are only given for pairs tested quantitatively and were modified based on the level of protein expressed as measured by Western Blot. For example, if a certain protein was expressed at a low concentration but its interaction with another protein allowed for growth on selective media even at a very high dilution factor, it was considered a strong interaction.

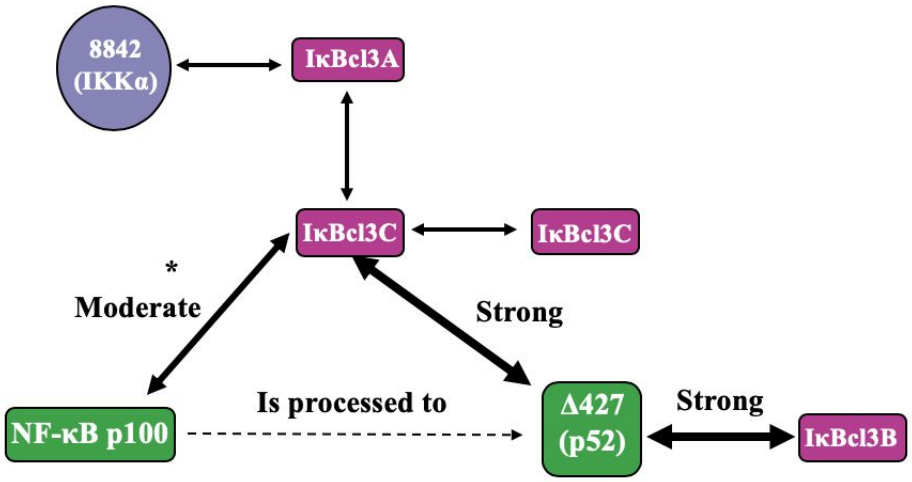


Figure 6. Summary of physical interactions between Aiptasia proteins observed in qualitative and quantitative YTH tests. Strength of interactions is relative, and Western Blots were used to verify expression levels of each Aiptasia protein in haploid yeast. * denotes uncertainty whether this interaction is occurring between full-length NF-κB or a C-terminally cleaved NF-κB, as was observed in the Western blot.

Discussion

This work explored the structure and interactions of the Aiptasia IκBcl3 proteins. Based on protein sequence homology comparisons and interactions in the Yeast Two Hybrid system, it remains difficult to determine with certainty whether any of the Aiptasia IκBcl3 proteins may be further classified as IκB homologs or Bcl3 homologs. However, I propose the following

pathway of NF-κB regulation in Aiptasia that incorporates what was observed in the present and in previous studies (Figure 7).

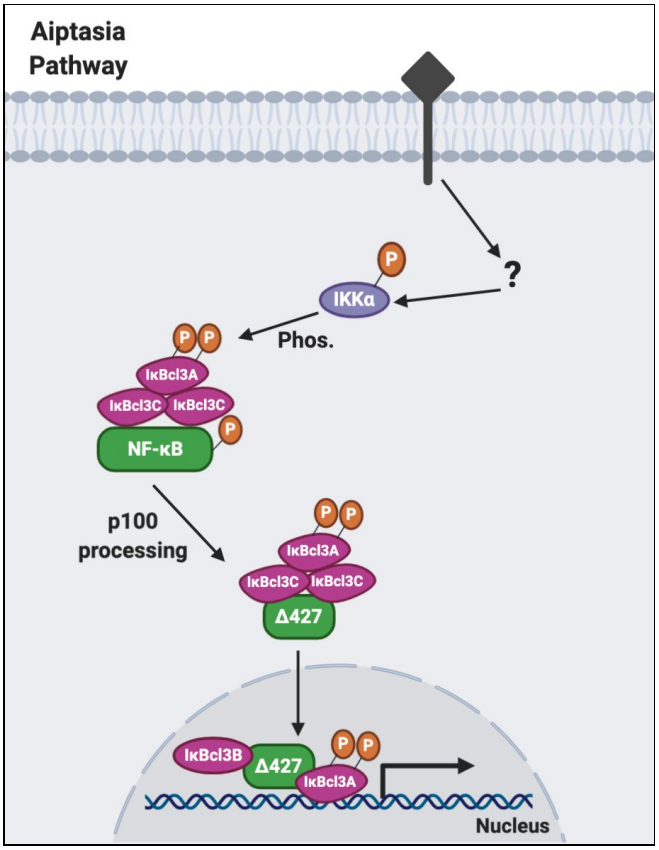


Figure 7. Hypothesized NF-κB signaling pathway in Aiptasia determined by sequence homology and protein-protein interactions. When the pathway is inactive, IκBcl3-A is in complex with IκBcl3-C and full-length NF-κB. Upon activation, the phosphorylated IKKα homolog phosphorylates IκBcl3-A at S38 and S43. This changes the conformation of the complex and full-length NF-κB may be processed to the active p52 homolog (Δ427), which binds to IκBcl3-B and IκBcl3-B to induce transcription of immune response genes.

In this mechanism, a phosphorylated IKKα homolog - whether in complex or as a monomer has yet to be determined - phosphorylates the N-terminus of AipIκBcl3-A at serines 38 and 43. When in the inactive state, AipIκBcl3-A is in complex with an AipIκBcl3-C homodimer and the AipNF-κB p100 homolog. Upon phosphorylation of AipIκBcl3-A, the complex undergoes a conformational change that allows AipNF-κB p100 to be processed to the active p52 form, which can then enter the nucleus. In the nucleus, p52 binds to target DNA sequences in complex with AipIκBcl3-A and AipIκBcl3-B, which may then induce transcription of immune

response genes. In this model, AipIκBcl3-A and AipIκBcl3-C are acting like IκB-like proteins during inactivation, and upon phosphorylation, AipIκBcl3-A transitions to function as a Bcl3-like protein with AipIκBcl3-B.

AipIκBcl3-A was included as a homolog for both IκBα and Bcl3 because of its ability to autoactivate and its homology to both protein subfamilies. As conserved domain analysis revealed, AipIκBcl3-A contains six ankyrin repeats, like human Bcl3 and opposed to the five ankyrin repeats present in human IκBα. Additionally, the ability of AipIκBcl3-A to activate transcription of reporter genes without the presence of the Gal4p activation domain demonstrates that this protein can recruit the cellular machinery necessary to induce transcription, a function of Bcl3 proteins. Because it could not be determined through YTH whether AipIκBcl3-A and AipΔ427 physically interact, it is likely that AipIκBcl3-A can function in the nucleus as a Bcl3-like protein to aid in transcription induction.

There are other caveats to the proposed pathway that require future research. As mentioned in the results, in addition to AipIκBcl3-A, AipNF-κB, and AipΔ427 were also autoactivators in YTH, meaning that the complete binding partner profiles of these three proteins could not be determined. For example, it was impossible to test through YTH whether these proteins interacted with one another or homodimerized. Additionally, it may be possible that the Aiptasia p52 homolog is sufficient to activate transcription, because AipΔ427 autoactivates in YTH, even though no canonical transactivation domain (TAD) was identified in the peptide sequence.

In order to validate or refute the claims supported by this study, further experimentation is necessary. The first step should be to assay the following interactions quantitatively, as data

gathered from these interactions was from only qualitative YTH: GBK-IκBcl3-C x GAD-IκBcl3-C; GBK-IκBcl3-C x GAD-IKK; GBK-IκBcl3-C x GAD-IκBcl3-A; GBK-IKK x GAD-IκBcl3-A. This is important as it was difficult to score growth of each diploid with such limited data (only one or two patches at an unknown concentration). Growth to saturation in liquid media will allow for either verification of a positive interaction or evidence that any interaction observed at the qualitative level was negligible. Next, co-immunoprecipitation experiments should be performed. Co-immunoprecipitation will support the protein-protein interactions between Aiptasia protein partners observed in YTH and will, importantly, allow the assessment of interactions that could not be tested because of autoactivation. Finally, immunofluorescence experiments in Aiptasia or in cell culture with tagged proteins or antibodies specific to each AipIκBcl3 will demonstrate any differences in intracellular localization between the three proteins; if any of the three proteins localize to the nucleus, they are more likely to function as Bcl3-like proteins, but if they localize to the cytoplasm, they are more likely to function as IκBα-like proteins. Colocalization with the Aiptasia p100 versus p52 homologs will also be observable through immunofluorescence experiments.

Furthermore, there are other analyses to further distinguish the three AipIκBcl3 as IκB or Bcl3 homologs. One avenue to explore is the mechanisms of turnover of IκB or Bcl3 proteins. It has been determined that a doublet of lysine residues N-terminal to S32 and S36 (K21 and K22) is required for IκBα degradation in humans (Huxford and Ghosh, 2009). After IKK phosphorylates S32 and S36, βTrCP E3 ubiquitin ligase recognizes the conserved phosphorylated sequence (DS*GXXS*) and polyubiquitinates K21 and K22, which targets IκBα to the proteasome (reviewed by Ben-Neriah, 2002 and Hayden and Ghosh, 2008). Human Bcl3

also undergoes a phosphorylation-dependent ubiquitination event (Wang et al., 2017). When Bcl3 S41 (S33 in Wang et al., 2017) is unphosphorylated, Bcl3 is tagged for proteasomal degradation via polyubiquitination to K56 (K48 in Wang et al., 2017). When S41 becomes phosphorylated via Protein Kinase B (PKB/Akt), ubiquitination switches from K56 to K71 and becomes a stabilization signal (Wang et al., 2017).

Analysis of the ubiquitination of conserved lysine residues across the N-termini of the three AipIκBcl3s may yield information that could allude to distinct degradation/stabilization mechanisms of the three proteins. Interestingly, AipIκBcl3-A contains two lysine residues (K26 and K29) approximately 10 residues upstream of the putative IKK phosphorylation site (S38 and S43), and the sequence containing these two lysines strongly resembles the conserved sequence of recognition by βTrCP E3 ubiquitin ligase: 37-DSGLGDS-43. The N-termini of AipIκBcl3-B and AipIκBcl3-C are relatively enriched in serine and lysine and share no obvious conserved sequences with human IκBα or human Bcl3 so it is more challenging to postulate the sites and roles of phosphorylation and ubiquitination for these two proteins. However, the function of specific lysine residues as targets for polyubiquitination within each AipIκBcl3 may be determined by synthesizing mutant AipIκBcl3 proteins with a target lysine mutated to alanine. The mutated protein may be co-transfected into a human cell line with either a constitutively-active IKK or Akt, then compared to a cell line co-transfected with the wild type AipIκBcl3 and constitutively-active IKK or Akt. Ubiquitination could be compared via immunoblotting against ubiquitin after immunoprecipitation of the AipIκBcl3 proteins.

Determination of the specific roles that each AipIκBcl3 protein plays within the NF-κB pathway, Aiptasia innate immunity, and symbiosis regulation necessitate genome editing

techniques - particularly knockdowns or knockouts. Knocking down specific AipIκBcl3s may tell us whether that protein had a stimulatory or inhibitory effect on NF-κB response genes or on the processing of NF-κB. As the NF-κB pathway is known to be down-regulated in symbiotic and upregulated in aposymbiotic Aiptasia, by inhibiting or constitutively inducing the NF-κB pathway, we may learn more about the interplay between maintenance/loss of symbionts and the immune response (Mansfield et al., 2017). Several laboratories across the world are working to solidify genome editing protocols via morpholinos and CRISPR/Cas9 in Aiptasia (Jones et al., 2018), in the non-symbiotic anemone *Nematostella vectensis* (Brennan et al., 2017; Layden et al., 2013) and even in a reef-building coral *Acropora millepora* (Cleves et al., 2019). The insights gained from these largely cellular and molecular methodologies will allow researchers to make strides into pinpointing mechanisms for coral rehabilitation and conservation that are based upon a more complete understanding of the unique biology of cnidarians.

Cell Biology and Conservation

Descriptive studies, such as the one presented here, are often hard to conceptualize as key to coral conservation efforts. However, such endeavors, in order to be completely successful, require an understanding of the intricate pathways that maintain the Symbiodiniaceae-cnidarian relationship and how such pathways are perturbed by stressors. This field of research - cellular and molecular studies of coral bleaching and disease - is vital to the global effort to protect coral reefs from further decimation. Understanding how the immune system is regulated differentially in cnidaria in order to maintain intracellular, obligate, commensals while at the same time protecting against infection by pathogens will not only provide avenues to monitor, rehabilitate,

and understand coral health at a deeper level, but may provide insight into the evolution of the immune system from basal metazoans to humans.

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