HBP1 promotes neuroblastoma differentiation by inhibiting MYCN transcription factor activity

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ABSTRACT

Neuroblastoma (NB) is a malignancy of the developing sympathetic nervous system and is the most common pediatric solid tumor. NB displays a wide range of clinical behavior: while many children have intractable disease that does not respond to standard therapy, infants frequently show spontaneous regression or differentiation of their tumors with minimal treatment. It is unclear what biological events drive this spontaneous regression, though current evidence implicates reactivation of normal differentiation pathways of sympathetic neuroblasts (SNs). The transcriptional regulator HBP1 has recently been shown to be highly expressed in favorable NB, particularly NB that is actively regressing. HBP1 is emerging as a general effector of terminal differentiation that acts in part by repressing expression and activity of c-myc. The c-myc homolog MYCN is highly correlated to biological behavior of NB and shares the structural features that allow HBP1 to act on c-myc. **I hypothesize that HBP1 promotes terminal differentiation of sympathetic neuroblasts and neuroblastoma cells by inhibiting MYCN transcription factor activity.** I will determine whether HBP1 is sufficient to drive differentiation of SNs and NB cell lines *in vitro* and whether HBP1 modulates sensitivity to differentiation-inducing agents. I will examine the role of HBP1 in endogenous SN differentiation in zebrafish. I will also use an established zebrafish model of NB to determine whether HBP1 overexpression induces NB regression. I expect that HBP1 overexpression will promote terminal differentiation of SNs; I also expect that induction of HBP1 overexpression will promote NB regression. I will test the hypothesis that HBP1 interacts with MYCN protein and determine how HBP1 affects expression of MYCN target genes by analyzing mRNA and protein levels. I expect that HBP1 will bind MYCN protein and inhibit transactivation of MYCN target genes. These studies will elucidate the link between SN differentiation and NB regression and may establish HBP1 as an important therapeutic target in refractory NB.

HYPOTHESIS: HBP1 promotes terminal differentiation of sympathetic neuroblasts and neuroblastoma cells by inhibiting MYCN transcription factor activity.

LONG TERM GOALS: This project aims to (1) understand the role of HBP1 in NB pathogenesis and prognosis; (2) explore the mechanisms of SN and NB differentiation; and (3) elucidate the links between SN differentiation and NB spontaneous regression.

SPECIFIC AIM 1 – To test the hypothesis that HBP1 promotes terminal differentiation of neuroblastoma cells in culture and in zebrafish. In part one, I will determine whether HBP1 overexpression is sufficient to promote differentiation of NB cell lines; I expect HBP1 will be sufficient to drive differentiation in the absence of other cues. I will also test how HBP1 overexpression or siRNA knockdown affect the sensitivity of NB cell lines to differentiation by retinoic acid and phorbol esters; I expect HBP1 overexpression and knockdown to make NB cells more sensitive and more resistant, respectively, to differentiation cues. In part two, I will generate a transgenic zebrafish that inducibly overexpresses HBP1 in SNs to determine how HBP1 affects differentiation of SNs *in vivo*. I will monitor the size and differentiation state of the SN population over 30 days in HBP1-expressing zebrafish and compare it to the SN population of untreated zebrafish. I expect HBP1 overexpression to accelerate SN differentiation. I will breed my transgenic model to a zebrafish model of NB to evaluate how HBP1 overexpression affects NB development. I expect HBP1 overexpression will induce significant regression of NB.

SPECIFIC AIM 2 – To test the hypothesis that HBP1 binds MYCN protein and inhibits MYCN transcription factor activity. In part one, I will test whether HBP1 interacts with MYCN protein by co-immunoprecipitation. In part two, I will determine whether HBP1 inhibits MYCN transcription factor activity. Using chromatin immunoprecipitation, I will determine whether HBP1 affects MYCN binding to DNA. I will measure the effect of HBP1 on MYCN target gene transcription by qRT-PCR.

BACKGROUND AND SIGNIFICANCE

NB can spontaneously regress or differentiate into a benign tumor

Neuroblastoma (NB) is a malignancy of the developing sympathetic nervous system. It is the most common solid tumor of childhood, accounting for 7-10% of pediatric cancer cases[1] and 15% of pediatric cancer-related deaths.[2] NB exhibits marked clinical heterogeneity: while many children present with disease that responds poorly despite aggressive therapy, NB also has the highest rates of spontaneous regression and differentiation of any cancer – between 10 and 100 fold greater than for any other human cancer[3]– and infants can often be safely treated with observation alone.[2]

Spontaneous regression and differentiation typically occur in a subset of NB patients under 18 months old at diagnosis. In this age group, both localized tumors[4,5] and disseminated disease with a specific metastatic pattern and favorable biology, known as NB 4S,[6,7] mature or regress in 30-95% of patients with observation alone.[8-11]

It is unclear what factors drive NB to mature or spontaneously regress, but current theories implicate reactivation of normal SN differentiation pathways in response to external developmental cues. Favorable NB overexpresses neuronal differentiation proteins compared to unfavorable NB,[17] and this overexpression is accentuated in NB that is actively regressing.[18] Differentiating and regressing tumors express a largely overlapping set of genes, and both kinds of tumors express both pro-apoptotic and neuronal differentiation proteins.

Myc family transcription factors play a key role in NB pathogenesis

The Myc transcription factor family is part of the core set of growth regulatory genes active in every proliferating cell. Both c-Myc and MYCN bind DNA sequences known as E-boxes as Myc/Max heterodimers to activate transcription and promote G1/S phase cell cycle progression.[19] MYCN and c-myc have highly similar biochemical properties, as they share identical domains, dimerize with the same partners, and bind the same DNA sequences.[19] (FIGURE 1A) MYCN can functionally replace c-myc when expressed from the

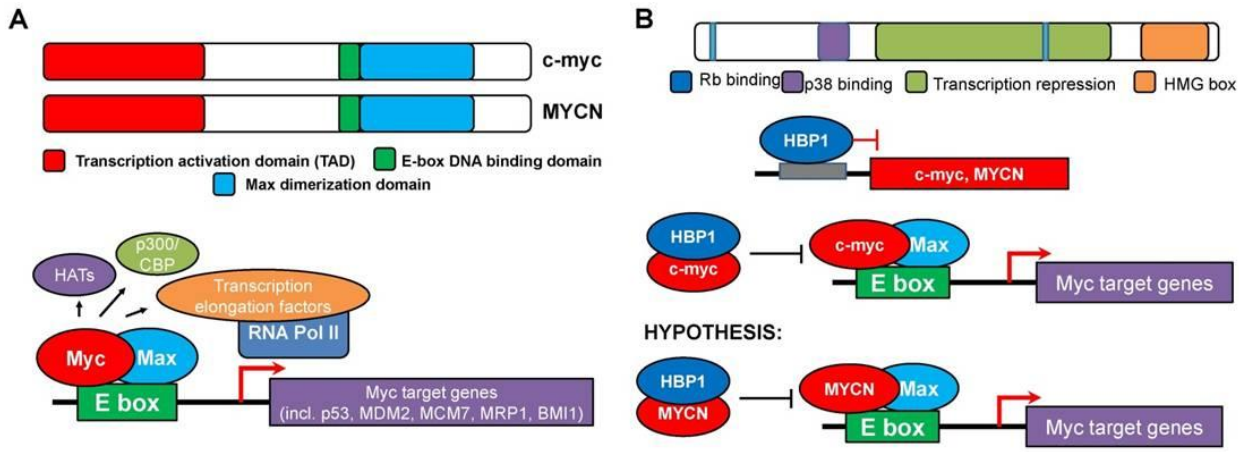

Figure 1. A) c-myc and MYCN are highly homologous and share domain architecture. Both Myc family transcription factors bind to E-box DNA sequences as Myc/Max heterodimers and promote transcription of an overlapping set of Myc target genes. **B)** HBP1 is a transcriptional repressor of c-myc and MYCN. HBP1 also regulates c-myc transcriptional activity by binding c-myc protein, preventing its interaction with E-box sequences. I hypothesize that HBP1 regulates MYCN transcriptional activity in a similar fashion to c-myc.

same locus, emphasizing their interchangeable characteristics.[20] The main difference between c-myc and MYCN is their expression pattern: while c-myc is expressed in all adult proliferating tissues, MYCN is expressed only in certain embryonic tissues, predominantly in the peripheral neural crest, the precursor tissue of SNs.[19]

Myc family transcription factors are important in NB pathogenesis. MYCN amplification is the biggest prognostic factor for poor outcome in NB and occurs in 25% of cases, almost none of which go on to differentiate or regress and all of which overexpress MYCN mRNA and protein.[21] Poor prognosis NB without MYCN amplification typically overexpresses c-myc and/or Myc family target genes.[22] Overexpression of MYCN in SNs is sufficient to produce NB in both mouse[23] and zebrafish[24] models. siRNA knockdown of MYCN leads to growth arrest, neuronal differentiation and apoptosis in various NB cell lines,[25] emphasizing the importance of MYCN in maintaining the neuroblast phenotype.

HBP1 is upregulated in actively regressing NB and may be a general regulator of differentiation

In two separate microarray studies designed to identify genes differentially expressed in stage 4 and 4S NB, HMG-box containing protein 1 (HBP1) was specifically upregulated in stage 4S tumors that were actively regressing.[17,18] However, these studies involved very small samples of tumors, and these results have yet to be validated on a larger cohort. HBP1 is an HMG-box transcription factor important in cell cycle control, proliferation arrest, and differentiation. Though few studies have been done on HBP1, its expression leads to cell cycle arrest,[26,27] and it is a regulator of differentiation in muscle,[26,28] skin[29] and myeloid cells.[30,31] HBP1 promotes differentiation in these cell types by transcriptionally regulating cell cycle inhibitors like p16[32] and p21[33] and cell cycle activators like cyclin D1.[34] In addition to being a transcription factor, HBP1 interacts with proteins involved in cell growth, proliferation and senescence. HBP1 function is tightly regulated by Rb[26-28] and p38,[35,36] and it can inhibit Wnt/β-catenin signaling by chelating TCF/LEF transcription factors.[34,37]

Given the evidence that HBP1 promotes differentiation in many cell types and appears to do so by acting through general cell cycle control factors, it has been suggested that HBP1 is a general regulator of cell differentiation. Together with evidence of selective HBP1 expression in regressing NB, these data suggest the hypothesis that HBP1 is involved in SN and NB differentiation.

HBP1 inhibits Myc family gene expression and c-myc transcription factor activity

Another mechanism by which HBP1 represses cell cycle progression and promotes differentiation is through regulation of Myc family members. HBP1 represses transcription from the MYCN promoter 5- to 15-fold[26] and represses transcription from the c-myc promoter 10-fold.[38] HBP1 also binds c-myc protein on its transcriptional activation domain, preventing c-myc binding to DNA and inhibiting its transcription factor activity independently of any change in c-myc protein levels.[38] (FIGURE 1B)

Because of the high similarity between c-myc and MYCN and the known interaction between HBP1 and c-myc protein, it is likely that HBP1 can also interact with MYCN and affect

transcription of MYCN target genes. The MYCN transactivation domain has high sequence homology with the c-myc transactivation domain, which is known to bind HBP1. Both the MYCN and c-myc transactivation domains bind identical DNA sequences and regulate similar genes, and so they would likely be similarly affected by HBP1. This supports the hypothesis that HBP1 binds MYCN and regulates MYCN target genes.

It is already known that HBP1 transcriptionally represses MYCN; however, in the context of MYCN amplification, as is frequently observed in neuroblastoma, MYCN transcriptional repression may have little effect on MYCN mRNA levels due to the large number of MYCN promoters in the modified genome. MYCN mRNA levels do not increase linearly with MYCN copy number and plateau around 10 copies of MYCN, while several dozen copies can routinely be seen in MYCN amplified tumors.[39] This suggests that protein-level regulation of MYCN transcription factor activity, rather than transcriptional regulation of MYCN mRNA and protein levels, may be more effective at controlling MYCN oncogenicity in NB. If HBP1 regulates MYCN protein activity, then therapies that induce HBP1 expression may be attractive in MYCN-amplified NB. Given that MYCN-amplified NB has an extremely poor prognosis even with modern multimodal therapy, new targets that regulate MYCN behavior are of high importance in NB treatment.

EXPERIMENTAL DESIGN

SPECIFIC AIM 1: To test the hypothesis that HBP1 promotes terminal differentiation of NB cells in culture and in zebrafish

Aim 1A: Does HBP1 promote differentiation in NB cell lines?

Rationale: NB cell lines are a classic model for sympathetic nervous system differentiation because their behavior in cell culture in response to chemical agents closely reflects neuronal development.[40] Because HBP1 overexpression is sufficient to initiate differentiation of other cell types, I hypothesize that HBP1 overexpression is sufficient to initiate differentiation of NB cell lines. If HBP1 is not sufficient to make cells differentiate, it may still make NB cells more

sensitive to differentiation-inducing agents. Retinoids like 13-*cis*-retinoic acid (RA) promote differentiation of many NB cell lines, including LA-N-5 and LA-N-1.[41,42] Phorbol esters, including 12-O-tetradecanoyl phorbol-13-acetate (TPA), promote differentiation in many other NB cell lines, including SH-SY5Y.[43] I hypothesize that HBP1 will reduce the concentration of RA or TPA required for inducing differentiation of NB cell lines.

Experiment 1A.1: Is HBP1 overexpression sufficient to induce differentiation of NB cell lines? Human HBP1 will be cloned into a pEF-BOS vector (Belgian Co-Ordinated Collection of Micro-Organisms), which drives constitutive overexpression of a target gene.[44]The NB cell lines SH-SY5Y, SK-N-AS (ATCC), LA-N-5 and LA-N-1 (a kind gift of Dr. R. Seeger, Children's Hospital of Los Angeles) will be transfected with pEF-BOS-HBP1 or an empty pEF-BOS vector as a negative control. I will verify HBP1 expression by immunocytochemistry with an HBP1 antibody (Santa Cruz). Every day for 8 days, 5 random 100x fields of cells will be examined by light microscopy to determine the percentage of cells with neurites longer than the cell body, a well-established marker of neuronal differentiation in NB cells.[42] (FIGURE 2) This time point was chosen because 8 days are sufficient for maximum differentiation to occur in response to RA and TPA in these cell lines.[45,46]

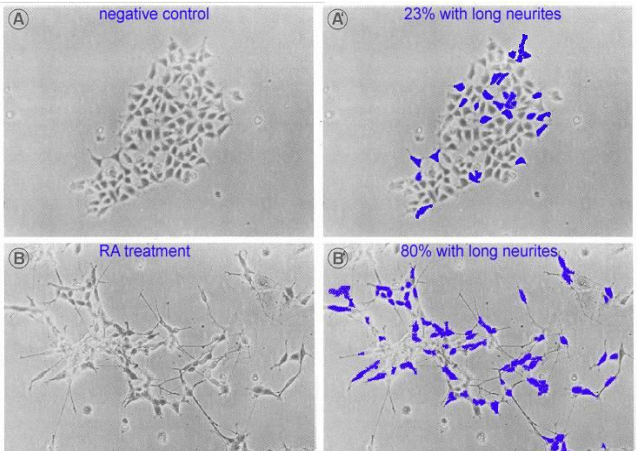

Figure 2. An example of morphological quantification of NB cell differentiation for experiments 1A.1 and 1A.2.Adapted from Haussler et al 1983.[60] **A)** LA-N-1 NB cells treated for 8 days with 1µM ethanol as a negative control. **A')** Cells with neurites longer than their cell body were quantified visually using Adobe Photoshop; 29 of 126 (23%) cells have neurites. **B)** LA-N-1 NB cells treated for 8 days with 1µM RA. **B')** Cells with neurites longer than their cell body were quantified visually using Adobe Photoshop; 70 of 87 (80%) cells have neurites.

Expected results: I expect that HBP1 overexpression will induce neurite extension, a morphological marker of neuronal differentiation, within 8 days of transfection.

Experiment 1A.2: How does HBP1 overexpression or knockdown affect NB sensitivity to differentiation by RA and TPA? I will overexpress HBP1 in the NB cell lines SH-SY5Y, SK-N-

Table 1. HBP1 transfections in NB cell lines.

Sample	Transfection	Experiments
HBP1 overexpression	pEF-BOS-HBP1 plasmid	1A.1, 1A.2
Overexpression (-) ctrl	pEF-BOS empty plasmid	1A.1, 1A.2
HBP1 knockdown	siRNA	1A.2
Knockdown (-) ctrl	Scramble siRNA	1A.2

AS, LA-N-5 and LA-N-1 as described above. I will knock down HBP1 by siRNA transfection (Santa Cruz) using the manufacturer's protocol and will use a scrambled siRNA (Santa Cruz) as a negative control. (TABLE 1) Beginning 24 hours after the initial transfection of the HBP1 plasmid or siRNA, I will treat the cells continuously with various doses ofRA (Sigma) dissolved in ethanol, TPA (Sigma) dissolved in ethanol, or ethanol alone as a negative control. (TABLE 2) Without HBP1 treatment, 1 µM RA and 3nM TPA should induce neurite formation in cells within 8 days. Every day for 8 days, 5 random 100x fields of cells will be examined by light microscopy to determine the percentage of differentiated neurons.

Drug	Carrier	Dosages	References
13-cis-retinoic acid (RA)	ethanol	0.1, 1, 10 µM	12, 60, 61
12-O-tetradecanoyl phorbol-13-acetate (TPA)	ethanol	0.3, 3, 30 nM	26
Ethanol (neg ctrl)	N/A	N/A	N/A

Table 2. Drug treatments for inducing differentiation in NB cell lines in experiment 1A.2.

Expected Results: I expect that HBP1 overexpression will either reduce the dose of RA or TPA required for differentiation or accelerate the onset of neurite extension. I expect that HBP1 knockdown will increase the dose of RA or TPA required for differentiation or inhibit differentiation altogether. (FIGURE 3)

Pitfalls and Alternatives: HBP1 has been reported to initiate cell cycle exit prior to terminal differentiation of several cell types. As such, HBP1 overexpression may interfere with cell growth. HBP1 may also impact NB cell viability. Most studies involving HBP1 overexpression in cell lines have not significantly impacted cell viability and have allowed for accurate measurements of differentiation over up to 10 days.[26-28,34] However, another study showed HBP1 overexpression significantly increased apoptosis in leukemic myeloid cells within 4 days.[31] If cells cannot be followed over 8 days due to loss of viability, we will follow cells until they drop below 75% viability as determined by Alamar Blue (Invitrogen) staining of a

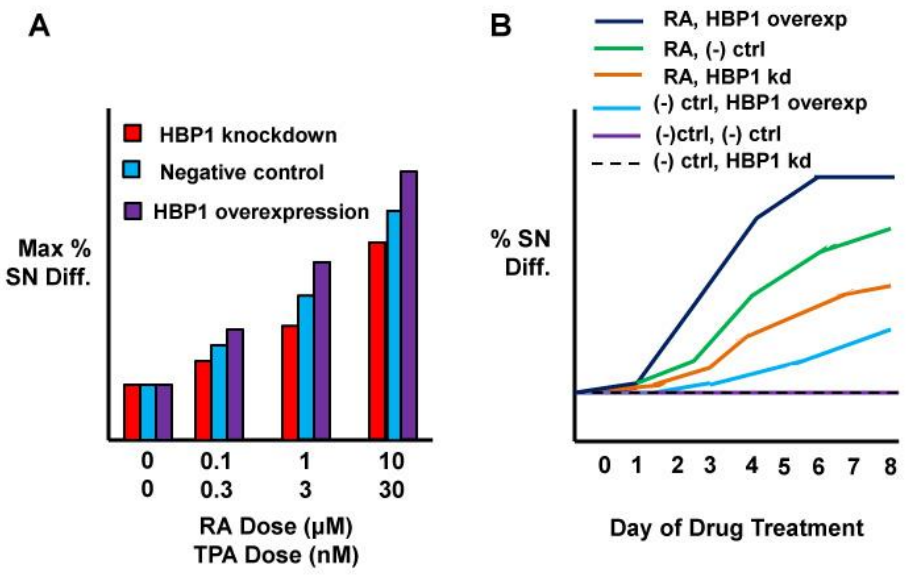

Figure 3. A) Expected SN differentiation after 8 days of RA or TPA treatment following HBP1 modulation. I expect RA and TPA will both increase SN differentiation in cell lines in NB cell lines in a dose-dependent manner. I expect that for all doses, HBP1 knockdown will reduce SN differentiation and HBP1 overexpression will increase SN differentiation. **B)** Expected time course of SN differentiation at a given dose of RA; results are expected to follow the same pattern at all doses for both drugs. HBP1 overexpression without drug treatment is expected to promote some differentiation (light blue), as is drug treatment without HBP1 modulation (green). HBP1 overexpression and knockdown are expected to increase or decrease, respectively, SN differentiation following drug treatment (dark blue, orange). All data are simulated.

representative sample of cells. Morphological differentiation is visible beginning 3 days following RA and TPA treatment; if cells survive for at least 3 days then this measurement can still be used. If cell viability drops before 3 days, then immunocytochemical (ICC) markers of differentiation will be used instead; markers like GAP43[53], neuropeptide Y[52], and neuron-specific enolase[42] are expressed as soon as 24 hours after induction of SN differentiation.

As discussed above, HBP1 affects differentiation in part through the MYCN transcription factor. This implies it could have different effects in MYCN-amplified cell lines, such as LA-N-5 and LA-N-1, and MYCN-nonamplified cell lines such as SH-SY5Y and SK-N-AS.[47] Since in this aim we are interested in the behavior of HBP1 alone rather than its relation to MYCN, we may need to consider only the data from SH-SY5Y and SK-N-AS cells if the data are significantly different in LA-N-5 and LA-N-1 cells. I expect that MYCN amplification will not significantly impact HBP1 effects because MYCN mRNA levels do not increase linearly with copy number,[39] but this could easily be incorrect as HBP1 is known to act in part through MYCN and it is unclear whether MYCN gene dosage or protein level is most important in determining the

effects of HBP1. Thus, this experiment may also provide some insight into the role of HBP1 in the context of MYCN amplification.

Aim 1B: Does HBP1 overexpression promote differentiation of SNs and reduce NB?

Rationale: Zebrafish are an excellent model for studying developmental phenotypes because they are cheap, small, have short generation times and lay dozens of eggs per day, are externally fertilized, and have transparent embryos and zygotes. This last feature makes them particularly amenable to serial imaging and makes it simple to trace a fluorescently labeled population of cells in live animals. Zebrafish can be genetically manipulated with high efficiency, and it is routine to generate stable transgenic lines. Using the Tol2 transposon system, I will generate a transgenic zebrafish line that inducibly overexpresses HBP1 under the SN-specific Dβh promoter. Using these transgenic fish, I will determine whether HBP1 promotes differentiation of SNs *in vivo*. I will then breed my HBP1 zebrafish with the Dβh:MYCN zebrafish NB model. I will allow fish to develop NB for 20 weeks then induce HBP1 expression to test whether HBP1 expression can induce NB regression *in vivo*. I hypothesize that HBP1 overexpression will accelerate SN differentiation and reduce SN population size in zebrafish; I further hypothesize that induction of HBP1 overexpression can induce NB regression in established zebrafish NB.

Prep step 1B.a: engineer Tol2[Dβh:mCherry;TetON-HBP1] plasmid and Tol2[Dβh:mCherry] plasmid: The Tol2 transposon system optimizes speed and efficiency of zebrafish transgenesis; over 50% embryos co-injected with a transgene plasmid and transposase plasmid demonstrate germline transmission of the transgene.[48] The Tol2 system is compatible with Gateway® cloning technology with freely available destination vector and transposase plasmid[49] (a kind gift of Dr. Koichi Kawakami, University of Utah). I will clone human HBP1 under the zebrafish Dβh promoter (Dβh:HBP1) into a pENTR 1A Dual Selection vector (Invitrogen) in between *XbaI* (New England BioLabs) restriction sites. To reduce the chance that HBP1 overexpression in SNs during embryogenesis could disrupt normal

development and prevent successful establishment of a transgenic line, I will also put HBP1 under control of a dually inducible Tet response element (TetON) to make HBP1 doxycycline- and dexamethasone-inducible (dox/dex) in addition to tissue-specific.[62] In this same vector, I will also include Dβh:mCherry. The mCherry marker serves two purposes: it will allow screening for embryonic transgene integration by fluorescence microscopy, and it will allow live measurement of SN number by fluorescence microscopy. As a control, I will clone an entry vector that includes only Dβh:mCherry, allowing baseline SN measurement but not modifying HBP1 status. I will then combine entry and destination vectors to create Tol2 expression vectors ready for embryonic injection.

Prep step 1B.b: Establish transgenic Dβh:mCherry and Dβh:mCherry;TetON:HBP1 zebrafish: Wild type AB zebrafish (Zebrafish International Resource Center) will be maintained by standard protocols.[50] Embryos will be collected and co-injected with transposase and eitherTol2[Dβh:TetON:HBP1;Dβh:mCherry] or Tol2[Dβh:mCherry], with 200-300 embryos in each branch. After 3 weeks, fish will be screened for mCherry expression and positive fish will be allowed to mature. When fish reach sexual maturity at 3 months, they will be bred and F1 progeny will be screened for stable transgene integration, resulting in Tg(Dβh:TetON: HBP1;mCherry) experimental and Tg(Dβh:mCherry) control zebrafish. These will be maintained as stable lines.

Pitfalls and Alternatives: Human HBP1 may not express properly in zebrafish. I will treat a sample of F1 fish with dox/dex by including the compounds in their water and evaluate HBP1 expression after 24 hours by immunohistochemistry. If I have trouble obtaining HBP1 expression in my transgenic fish, I will create an entry clone using zebrafish HBP1 and repeat the transgenesis. I choose to begin with human HBP1 despite the risk of nonfunctional transgenesis in the interest of consistency, as the rest of my experiments in this proposal use human HBP1, and because these experimental results would be more directly relevant to human NB. The Dβh promoter may also fail to express mCherry and/or HBP1 at a strong

enough level to produce a phenotype; this is unlikely, as the Dβh promoter can drive high level overexpression of EGFP-MYCN in SNs,[24] however due to peculiarities of cloning and gene expression it is possible. The TH promoter can also be used to drive SN-specific expression;[23] if mCherry and/or HBP1 fail to express under Dβh I will engineer them under TH.

Experiment 1B.1: How is SN population size and differentiation rate affected by HBP1 overexpression? This experiment will test whether HBP1 promotes neuronal differentiation of sympathetic precursor cells *in vivo*. The SN population size and SN differentiation rate will be compared in 100 Tg(Dβh:mCherry) negative control fish and 100 Tg(Dβh:TetON:HBP1;mCherry) experimental fish. Both fish populations will be raised in water containing dex/dox. Every 3 days during days 3-30 post-fertilization, I will measure the SN population size in each live fish by quantitative fluorescence microscopy. The SN population size will be directly proportional to the amount of whole-body mCherry fluorescence. Every 3 days, I will sacrifice 10 fish from each sample, fix and section them as described in Macdonald 1999,[51] and perform immunofluorescence (IF) microscopy for markers of neuronal differentiation, including neuron specific enolase[42] (Abcam), neuropeptide Y[52](Abcam), and GAP43[53] (Abcam) (secondary Invitrogen Alexa Fluor® goat anti-rabbit). I will examine 5 random fields containing at least 10 cells that express mCherry and are therefore SNs or sympathetic neurons.

Expected results: I expect HBP1 overexpression to decrease the SN population size and speed up SN differentiation in zebrafish.

Alternative Outcomes: It is also possible that HBP1 overexpression could paradoxically increase the SN population size; a large factor determining final neuron number is apoptosis, which can occur before the onset of SN differentiation,[54] and HBP1 overexpression could allow more cells to differentiate before apoptosis ensues.

Experiment 1B.2: Does HBP1 overexpression induce NB regression in zebrafish?: Tg(Dβh:EGFP-MYCN) NB model zebrafish (a kind gift of Dr. A. Thomas Look, Harvard University) will be bred with Tg(Dβh:TetON:HBP1;mCherry) and Tg(Dβh:mCherry) zebrafish to

Table 3. Transgenic zebrafish lines used in aim 1B.

Zebrafish Genotype (all Tg under Dβh promoter, HBP1 inducible under TetON)	SN fluorescence	Experiment	Sample role
mCherry	Red	1B.1	Negative control
mCherry; HBP1	Red	1B.1	HBP1 experimental
mCherry; EGFP-MYCN	Red, green	1B.2	Positive control
mCherry; EGFP-MYCN; HBP1	Red, green	1B.2	HBP1 experimental

generate NB model zebrafish with and without inducible HBP1 overexpression. (TABLE 3) Zebrafish will be raised by standard protocols in water without dox/dex. After 20 weeks, I expect 20% of zebrafish of each genotype to have developed NB as in Zhu et al.[24] NB will be defined as a tumor large enough to be seen without the aid of fluorescence microscopy, as in Zhu et al.[24] At this point, NB-negative fish will be sacrificed. NB-positive fish will be imaged by fluorescence microscopy and NB-positive fish of each genotype will be randomly divided into two treatment groups. One group will receive dox/dex in their water, while the other group will receive standard water. Once per week for 20 weeks, tumors will be imaged by fluorescence microscopy to monitor for tumor regression. I expect that the tumor burden of HBP1-positive fish treated with dox/dex will decrease over time, indicating NB regression. I expect that no other treatment groups will show significant NB regression.

Alternative Experiments: HBP1 may fail to induce regression but may instead induce tumor differentiation. Fish will be sacrificed at the end of the 20 week observation period and their tumors will be analyzed by IF microscopy for markers of neuronal differentiation. HBP1 induction of either regression or differentiation will be considered a favorable outcome, as they are currently thought to be regulated by similar processes in NB.

SPECIFIC AIM 2: To test the hypothesis that HBP1 binds MYCN protein and inhibits MYCN transcription factor activity

Aim 2A: Does HBP1 interact with MYCN protein?

Rationale: HBP1 is known to interact with c-myc protein, and MYCN is structurally homologous to c-myc. This suggests HBP1 may also interact with MYCN. I will test this hypothesis by co-immunoprecipitation (Co-IP).

Experiment 2A: Co-IP of HBP1 and MYCN: An antibody against HBP1 (described above), an antibody against MYCN (Thermo Scientific), and a negative control antibody for the transcription factor Sp1[38] (Santa Cruz) will be conjugated to protein G sepharose beads (Invitrogen). I will lyse SH-SY5Y cells and run the lysate through a column filled with anti-HBP1 beads, anti-MYCN beads, anti-Sp1 beads as a negative control, or plain protein G sepharose beads as a negative control. I will elute the proteins from the beads and run a Western blot, then probe the blot with various antibodies to determine whether certain proteins were pulled down.

I will determine whether c-myc (antibody: Santa Cruz) was pulled down with HBP1 as a positive control for HBP1 binding.[38] I will then ask whether MYCN was pulled down with HBP1. I will determine whether Max (antibody: Santa Cruz) was pulled down with MYCN as a positive control for MYCN binding.[19] I will then ask whether HBP1 was pulled down with MYCN. I will ensure that neither HBP1 nor MYCN was pulled down with the negative controls. [FIGURE 4]

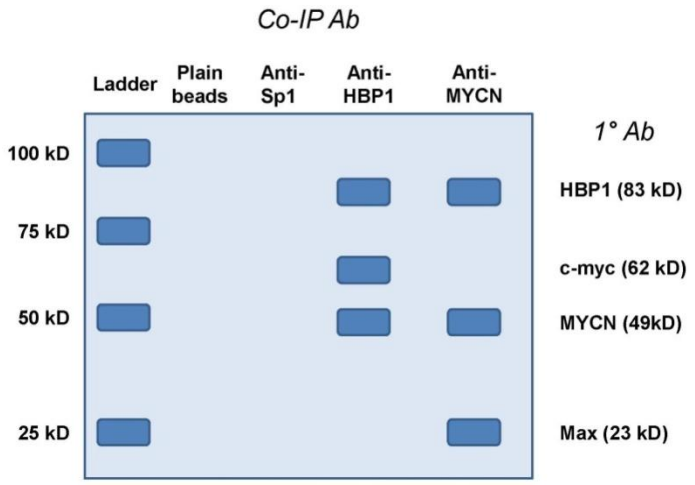

Figure 4. Simulated Western blot data for HBP1 and MYCN co-immunoprecipitation. As positive controls, HBP1 should pull down c-myc and MYCN should pull down Max. As negative controls, plain beads and Sp1 should not pull down any probed proteins. I expect HBP1 to pull down MYCN and MYCN to pull down HBP1.

If HBP1 interacts with MYCN, it would be interesting to learn which regions of MYCN and HBP1 are required for this interaction. I expect that the C-terminal of HBP1 and the transcriptional activation domain of MYCN would be required for this interaction, since these

regions are required for the HBP1/c-myc interaction. This would be an intriguing follow-up question that could be tested by deletion analysis. If HBP1 does not interact with MYCN protein, it may still affect MYCN regulation of target genes indirectly and MYCN transcriptional effects should still be measured. HBP1 may also act through a mechanism independent of MYCN.

Aim 2B: Does HBP1 inhibit MYCN-induced transcription?

Rationale: HBP1 is known to inhibit c-myc-induced transcription, and c-myc is structurally homologous to MYCN. This suggests HBP1 may also inhibit MYCN induced transcription. I will test this hypothesis by chromatin immunoprecipitation (ChIP) and qRT-PCR. To determine the effects of HBP1 on MYCN transcription factor activity, I will transiently transfect SH-SY5Y cells with pEF-BOS-HBP1 overexpression vector, empty pEF-BOS as a negative control, siRNA against HBP1, or a scramble siRNA negative control, as described above.

Experiment 2B.1: Does HBP1 inhibit MYCN binding to DNA? I will cross-link DNA and proteins by treating cells with 1% formaldehyde, then pellet and lyse the cells, sonicate the lysate to shear the DNA, and add anti-MYCN antibody; I will also include a no-antibody negative control. MYCN and bound DNA will be collected with a salmon sperm DNA/protein A agarose slurry (Millipore). After washing, protein-DNA complexes will be eluted from the antibody and the crosslinks will be reversed with heat. Using qPCR, I will evaluate the amount of MYCN binding to promoters of the MYCN target genes p53,[55] MDM2,[56] MCM7,[57] MRP1[58] and BMI1.[59] The amount of MYCN binding to target gene promoters will be compared in negative control, HBP1 overexpression, and HBP1 knockdown cells.

Expected Results: I expect that HBP1 overexpression will reduce MYCN binding to target gene promoters and HBP1 knockdown will increase MYCN binding to target gene promoters.

Pitfalls: Not all MYCN target genes may interact with MYCN in a given scenario due to chromatin state or interactions with other proteins. I will most strongly consider results from promoters that strongly bind MYCN in the negative control but bind MYCN at a significantly lower level when HBP1 is overexpressed.

Experiment 2B.2: Does HBP1 affect mRNA levels of MYCN target genes?: I will pellet and lyse the cells, then collect total RNA with an RNeasy Mini Kit (Qiagen) and convert the mRNA to cDNA using an RT^2 Easy First Strand cDNA Kit. The amounts of various MYCN target gene cDNAs (p53, MDM2, MCM7, MRP1, BMI1, and β-actin as a negative control) will be evaluated by qRT-PCR with SYBR Green based RT^2qPCR Primer Assays (Qiagen).

Expected Results: I expect that HBP1 overexpression will reduce MYCN target gene mRNA expression and HBP1 knockdown will increase MYCN target gene mRNA expression.

Pitfalls: Not all MYCN target genes may be transcribed in a given cell due to epigenetics or cell signaling mechanisms. I will most strongly consider results from cDNAs that are highly expressed in negative control cells and have a high fold-decrease in HBP1-overexpressing cells.

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