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Investigating downstream effectors of Yap1 mediated tumorigenesis

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Cindy Chang

We, the thesis committee for the above candidate for the
Master of Arts degree, hereby recommend
acceptance of this thesis.

Scott Lowe – Thesis Advisor
Professor, Department of Genetics, Stony Brook University,
Cold Spring Harbor Laboratory

Senthil Muthuswamy - Chairperson of Defense
Assistant Professor, Department of Genetics, Stony Brook University,
Cold Spring Harbor Laboratory

This thesis is accepted by the Graduate School.

Lawrence Martin
Dean of the Graduate School

Abstract of the Thesis

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Yap1 is a transcription factor that was identified by our group in 2006 as a potent oncogene influential in the development and maintenance of murine and human hepatocellular carcinoma (Zender, Cell 2006). By overexpressing wild-type and deletion mutants of the Yap1 gene, I determined that the transcriptionally active C terminus was essential for Yap1 to induce cellular transformation *in vitro* and tumor formation *in vivo*. Recent work by another group demonstrated that Yorkie, the Drosophila homolog of Yap, activates the expression of a microRNA called *bantam*, and that *bantam* alone is capable of the pro-growth effects of Yorkie overexpression (Thompson et al., Cell 2006). Although the *bantam* microRNA does not have a mammalian homolog by sequence homology, it is possible that Yap upregulates a microRNA in mammals that activates similar growth-promoting pathways. One of the aims of my thesis project was to identify these potential microRNAs. To this end, I developed a tet-inducible system to study the effect of Yap overexpression in tumor cells *in vitro* and *in vivo*. Mouse and human tumor cell lines were retrovirally transduced with tet-inducible Yap1, and the RNA of doxycycline induced vs. uninduced cells were analyzed by microarray for upregulated genes.

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Introduction and Specific Aims

Hepatocellular carcinoma (HCC), the most common type of liver cancer, is also one of the most common lethal cancers worldwide. The 5-year survival rate of patients diagnosed with HCC is only 8.9% (Farazi, 2006). The prognosis for patients with HCC is worsened by liver dysfunction caused by tumor progression. There are limited treatment options for HCC beyond surgical resection, which is only available in the early stages of disease, although the results of recent clinical trials have allowed FDA approval of sorafenib, a Ras kinase and VEGFR-2 inhibitor, for the treatment of HCC (Lang, 2008). HCC is therefore an example of a disease that urgently requires the development of effective drug therapies. Understanding the molecular mechanisms behind the initiation and development of cancers like HCC is essential to the design of rational drug therapies for their treatment.

We identified Yap1, or Yes-associated protein 1, as an oncogene responsible for HCC development when we performed high-resolution array-CGH platform (ROMA)-based comparisons of murine and human hepatocellular carcinomas (Zender et al, 2006). The ROMA results identified a common amplicon on 9qA1 in mice and the syntenic region, 11q22, in humans. The 11q22 amplicon is also amplified in other epithelial tumors, including esophageal, lung, and pancreatic carcinomas, indicating that the driving oncogenes of this amplicon can be important contributors to tumor development. We validated Yap1 and cIAP1 as the driving oncogenes in this amplicon, and used classic in vivo transformation assays to further demonstrate that Yap1 alone can rapidly induce tumor development.

In agreement with our findings, recently published literature shows that overexpression of Yorkie, the Drosophila homolog of Yap, leads to overproliferation mediated by cyclin E and the microRNA bantam (Cohen et al., 2006, Huang et al., 2005). However, earlier in vitro studies of mammalian cells describe Yap1 as an essential mediator of p73-dependent apoptosis and identify Yap1 as a potential tumor suppressor. It is therefore unclear how and in which contexts Yap1 may induce apoptosis and when it may stimulate proliferation. Clarification of Yap1 function will not only aid our understanding of an important oncogenic network, but it will help us identify therapeutic targets for tumors driven by the 11q22 amplicon. For these reasons, I proposed to investigate the role of Yap1 in tumorigenesis through identification of the downstream effectors of Yap that mediate its effect. My specific aims were as follows:

Aim I. Establish the functions of Yap1 relevant to tumorigenesis through:
- A. Characterization of the cellular response to Yap1 overexpression;
- B. Analysis of the structure and function of the Yap1 protein.

Aim II. Identify downstream targets of Yap1, through:
- A. Comparative microarray expression analysis of Yap1-upregulated genes in relevant cell types
- B. Functional validation of relevant targets.

Aim III. Design and test treatment strategies for 11q22 amplicon-containing tumors by targeting
- Yap1-activated effectors.

This report is a summary of the work completed in pursuit of these aims.

Background and Significance

Cancer is a set of diseases characterized by uncontrolled cellular proliferation and metastatic potential. The genetic alterations leading to cancer are multiple and varied: even when tumors arise from the same tissue, they can originate from different genetic lesions. Importantly, cancer cells are often "addicted" to their transforming lesion, meaning that loss of an oncogenic signal can cause cancer cells to die and tumors to regress (Weinstein, 2002). Thus, by classifying cancers according to their addicting lesions, we can potentially design treatment strategies tailored to each cancer.

To this end, our lab sought to identify commonly amplified genomic regions in hepatocellular carcinoma, a common and lethal cancer that does not currently respond to chemotherapy. We used Representational Oligonucelotide Microarray Analysis (ROMA), an array-based comparative genomic hybridization technology, to analyze mouse and human cancer samples. During this analysis we identified a common amplicon on 9qA1 in mice and on 11q22, the corresponding region in humans (Zender et al., 2006). Other researchers also identified 11q22 as a frequently amplified region in other epithelial tumors, such as mammary, oral, esophageal, lung and pancreatic carcinomas (Overholtzzer et al., 2006, Snijders et al., 2005, Imoto et al., 2001, Dai et al., 2003 and Bashyam et al., 2005). The recurrence of this amplicon suggests that one or more genes amplified within this region can act as a potent driving force in tumorigenesis. In agreement with data published by other groups, we identified cIAP1 as one of the driving oncogenes in this amplicon. However, we were the first to also identify Yap1 as another potent oncogene. We further established that overexpression of Yap1 alone can initiate tumorigenesis, and that this effect may be partly mediated by Yap1-induced cyclin E expression (Zender et al., 2006).

Despite the important role that Yap1 overexpression plays in cancer, there has been relatively little work characterizing the mechanism by which Yap1 mediates tumorigenesis. Yap1, or Yes-associated protein 1, was originally identified in chicken as a 65-kDa binding partner of the Src kinase family member, c-Yes kinase (Sudol, 1994). Yap1 was shown to bind to the SH3 domain on Yes, and with less affinity to other SH3 domain-containing molecules, through a proline-rich motif conserved in chicken, mouse and human homologs of Yap.

Investigators also discovered that Yap contained a novel protein-protein interaction domain near its N terminus. This domain, which they called the WW domain for the two conserved tryptophans that flank the sequence, was discovered when researchers noticed an imperfectly duplicated 38 amino acid sequence on mouse Yap1 that is not duplicated in human or chicken homologs (Bork et al., 1994; Sudol et al., 1995). A subsequent study revealed that the WW domain is prevalent among cytoskeletal and adaptor proteins, and that it binds proline-rich PPxY or PPLP motifs similar to, but distinct from, the motifs bound by SH3 domains (Sudol et al., 1995). Among other interactions, the WW d\omain suggests a cytosolic role for Yap1 as a mediator of retroviral budding (Garnier et al., 1996).

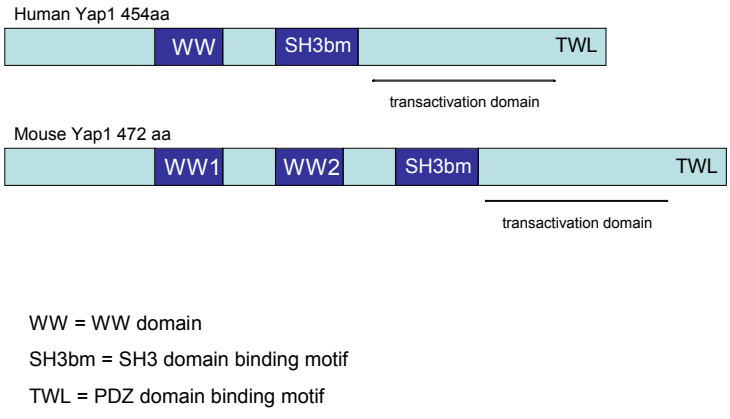

Figure 1: Functional domains on Yap1

Yap1 has also been shown to bind PDZ domains through a conserved TWL sequence on its extreme C terminus, which in airway epithelial cells causes Yap1 to localize exclusively to the apical compartment of the cytosol (Mohler et al., 1999). In these cells, Yap1 binds with high affinity to the second PDZ domain of the membrane-associated protein EBP50/NHERF, and is responsible for incorporating Yes kinase into the EBP50/NHERF complex. Given these and other data showing that Yap1 localizes to the cytosol, and since Yap1 lacks an obvious catalytic domain, researchers originally suggested that Yap1 function as a cytosolic adaptor protein (Sudol et al., 1995). The discovery that Yap1 contains a strong intrinsic transcription activation domain near its C terminus (Yagi et al., 1999), and that Yap1 can bind to PPxY motifs on transcription factors through its WW domain, modified this view to include a role for Yap1 in the nucleus. These characteristics suggested that Yap1 function might overlap with Taz, a protein with which Yap1 shares about 45% sequence homology. Both proteins are ubiquitously expressed, and Taz also has a WW domain, binds PDZ domains and demonstrates transcriptional activity (Kanai et al., 2000). However, mice homozygous for a targeted disruption in Yap1 were found to be embryonic lethal (Morin-Kensicki et al., 2006), suggesting first that Yap1 is essential for the development of the organism, and second, because Taz is unable to compensate for the loss of Yap, that Yap1 and Taz perform non-overlapping functions in the cell.

Much of the additional work describing Yap's role in mammalian cells focuses on its function as a transcriptional coactivator. In apparent contrast to what our data suggests, early reports indicated that Yap1 might play a role in mediating p73-activated apoptosis. Yap1 binds to members of the p53 family that have an intact PPxY motif (Strano et al., 2001). Overexpression of Yap1 enhances p73 transcriptional activity, and increases p73 activation of pro-apoptotic genes Bax and p53AIP1 in a PML-dependent manner (Strano et al., 2005). Yap1 binding stabilizes p73 (Levy et al., 2006). Akt phosphorylation of Yap1 reduces Yap1 enhancement of p73-mediated apoptosis (Basu et al., 2003); this suppression of Yap by Akt is thought to be an additional mechanism by which Akt acts as an oncogene. These events are dependent on a DNA damage signal (Strano et al., 2005).

However, it is important to note that most of these studies looked at the behavior of Yap *in vitro*. Recent *in vivo* work has shown that Yorkie, the Drosophila homolog of Yap, can promote cell proliferation. The Hippo/Warts pathway had been previously described in Drosophila as a tumor suppressor pathway, and Yorkie was identified as the downstream pro-proliferative target that Hippo and Warts suppressed (Huang et al., 2005). Yorkie is required for normal tissue growth in Drosophila, and was found to activate the transcription of cyclin E and DIAP1, an anti-apoptotic gene in Drosophila. This is interesting in light of our data, which show that mammalian Yap1 also induces cyclin E expression, and that Yap1 is co-amplified in tumors with cIAP1, a mammalian caspase inhibitor. Interestingly, cyclin E and DIAP1 expression were shown to be only partly responsible for the tissue overgrowth mediated by Yorkie. Recent studies have demonstrated that Yorkie activates the expression of a microRNA called *bantam*, and that *bantam* alone is capable of recapitulating the effect of Yorkie overexpression (Thompson and Cohen, 2006). Taken together, these results support a role for Yap1 as a potent pro-proliferative oncogene.

It is important to note here that although Yorkie contains a WW domain and a C-terminal transactivation domain, and shares high sequence homology with chicken and mammalian Yap1, Yorkie lacks the proline-rich SH3-domain binding motif and does not associate with Src kinase family members. Deletion of the SH3 binding motif in mammalian Yap1 may compromise its cytosolic functions, but does not hinder its ability to activate transcription (Yagi et al., 1999). This raises the question: does mammalian Yap1 require Yes/Src regulation or cytosolic function to mediate tumorigenesis? Furthermore, although it was previously known that mammalian homologs of Hippo and Warts can substitute for loss of the homologous genes in Drosophila, it has only recently been established that a connection in mammalian cells between the Hippo/Warts pathway and Yap1 exists (Dong et al., Cell 2007). The tumor suppressor Hippo

pathway functions to regulate Yap1 nuclear localization: activation of the Hippo pathway results in phosphorylation of Yap at S127 and cytosolic sequestration, whereas attenuation of Hippo activity allows Yap to accumulate in the nucleus and transactivate genes.

The aim of my proposed work was to understand how Yap1 mediates tumorigenesis. To this end, I analyzed the structure and function of Yap1 both *in vitro*, through cultured cell lines, and *in vivo*, using the mouse models of liver cancer previously established in our lab. I also sought to isolate the downstream effectors of Yap1 that are relevant for mediating tumorigenesis. The answers gained through this study would not only aid us in our understanding of an important oncogenic signaling pathway, but also help us design rational therapies for Yap1-initiated tumors.

Research Progress and Results

In order to identify chemotherapeutic targets specific to tumors harboring the 11q22 amplicon (11q22+ tumors), I sought to understand how Yap works as an oncogene and identify the downstream effectors of Yap. This work was divided into three specific aims:

Aim I. Structure and function analysis of the Yap1 gene.

I-1. Yap1 overexpression transforms NIH 3T3 fibroblasts.

After Yap1 was identified as a potential co-driving oncogene in the 11q22 amplicon, we found that Yap alone was capable of accelerating tumorigenesis in the genetic context in which the amplicon had first been found: p53 null, c-myc overexpressing hepatoblasts (Zender et al., 2006). We next asked whether Yap could initiate tumorigenesis without c-myc overexpression. This was done using a classic test for oncogenicity, in which Yap was overexpressed in NIH 3T3 fibroblasts or immortalized hepatocytes, and the cells were injected subcutaneously into nude mice. NIH3T3 fibroblasts have lost wild-type expression of the tumor suppressor $p16^{Ink4a}/p19^{Arf}$

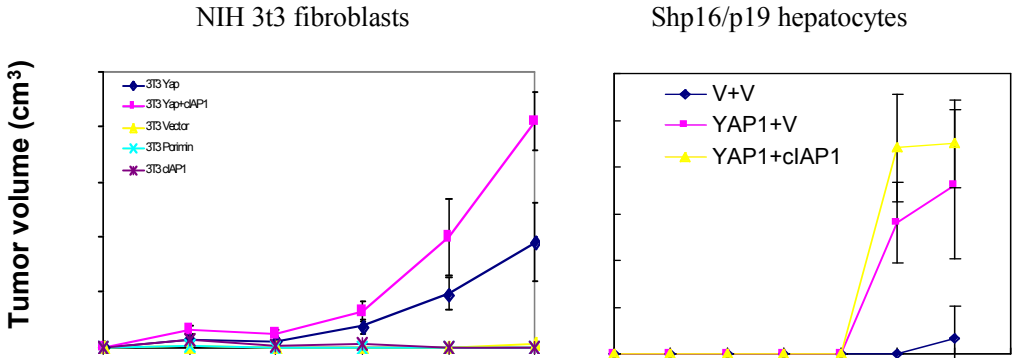

Figure 2: Yap1 alone induces tumor growth

locus, so we chose to compare them with hepatocytes that had been immortalized through stable expression of a short hairpin against $p16^{Ink4a}/p19^{Arf}$ (shp16/p19 hepatocytes). Tumor volume was measured over time (see Figure 1 below). We found that while cells co-expressing Yap and cIAP1, another gene amplified in the 11q22 amplicon, rapidly developed tumors in nude mice, cells expressing Yap alone were also capable of rapidly developing tumors. On the other hand, cells expressing cIAP1 alone developed tumors only after a very long latency. Therefore, Yap is a potent oncogene capable of initiating tumorigenesis.

I-2. Yap1 upregulates expression of cell cycle promoting genes.

To further investigate the ability of Yap to drive tumor formation, we looked at the gene expression profile of Yap-overexpressing cells. NIH 3T3 cells were transduced with a retrovirus containing either empty vector (MSCV) or MSCV-Yap. In the MSCV vector, Yap is overexpressed from the LTR promoter.

Total RNA from these cells was labeled with fluorescent dye and hybridized to a microarray, and gene expression between the two samples was compared. In confirmation of

Table 1: Yap1 induction of cell-cycle genes

Gene	Fold Induction
Cdc7	43
Cyclin E2	**41**
Cdc6	35
Orc1	29
Mcm2-7	4-15
E2F7	12
Orc2	8
Cyclin E1	8

our results from the *in vivo* tumor formation assays, we found that Yap upregulates the expression of genes that promote cell cycle progression. Interestingly, one of the upregulated genes was cyclin E2, a G1 cyclin that is specifically upregulated in tumor cells (Gudas et al., 1999). These data not only confirm Yap as an oncogenic stimulus in the cell, but identify potential therapeutic targets in Yap-driven tumors.

I.3. Human Yap1 constructs are tumorigenic.

Initial work on Yap-mediated tumorigenesis was obtained using mouse Yap1, which differs from human Yap1 by having an additional WW domain that researchers believe may bind different proteins than the first. Since I am ultimately interested in the oncogenicity of human Yap in human tumors, I next examined the tumorigenicity of human Yap and created several deletion mutants of human Yap for comparison.

I cloned the full mouse and human Yap cDNAs into a retroviral vector, using the MSCV backbone with a puromycin resistance gene driven by the PGK promoter, followed by IRES-GFP to visually mark the cells (MSCV-PIG). I then subcloned an initial series of Yap deletion mutants into this vector (Figure 2). Two of the mutants, ΔWW and ΔC, were obtained from Giovanni Blandino and subcloned into MSCV-PIG using PCR. The ΔWW mutant lacks residues 175-187 and 189-203, which encompass conserved regions in the WW domain; the ΔC deletion terminates the Yap1 protein after residue 264. I also created another deletion mutant (ΔSH3bm) lacking residues 240-249, which loses the SH3 domain binding motif conserved in chicken and mouse Yap homologs ΔSH3bm mutant. The sequences of these constructs were verified, and protein expression was visualized by Western blot (see Figure 4).

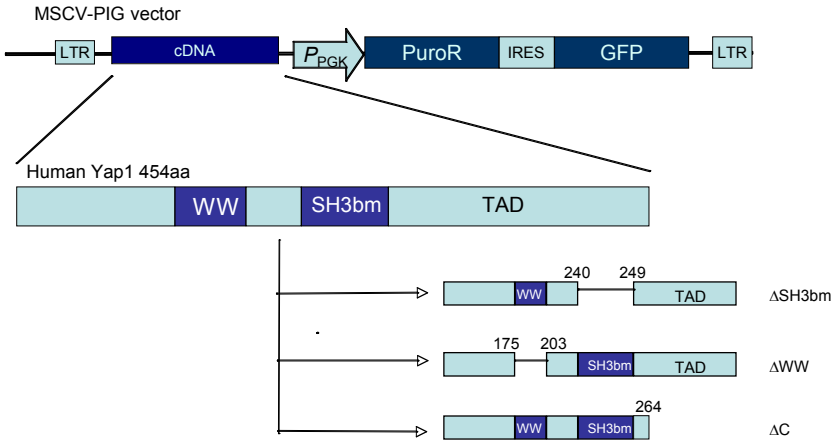

Figure 3: Human Yap1 deletion constructs

These wild-type and mutant Yap1 constructs were then infected into NIH 3T3 fibroblasts, and the infected cells were selected with puromycin and injected subcutaneously into nude mice at 200,000 cells per injection site. It is important to note that this is much less than the 2-3 million cells routinely used in our

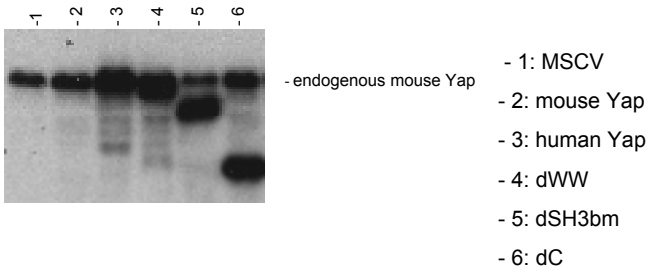

Figure 4: Human Yap1 deletion constructs

lab for *in vivo* tumorigenicity assays. I chose to inject the cells at a lower count because I could not expand the population of cells infected with ΔC-Yap, which seemed to either undergo cell cycle arrest after infection or grow much more slowly than cells infected with the MSCV vector alone.

My first observation was that during puromycin selection, NIH3T3 cells expressing wild-type, ΔWW or ΔSH3bm Yap constructs all developed a spindle-like morphology typical for a transformed cell (not shown). NIH3T3 cells infected with MSCV or ΔC-Yap did not change morphology. Next, tumors developed by 4-5 weeks in mice injected with cells overexpressing wild type Yap, ΔWW-Yap, or ΔSH3bm-Yap, testifying to the strength of Yap as an oncogene (see Figure 5; ΔSH3bm not shown). However, cells infected with MSCV alone or ΔC-Yap did not develop tumors at all. These results were replicated for all construct in 3 separate experiments, using 2 mice per construct, and 2 injections per mouse (12 potential tumor sites total).

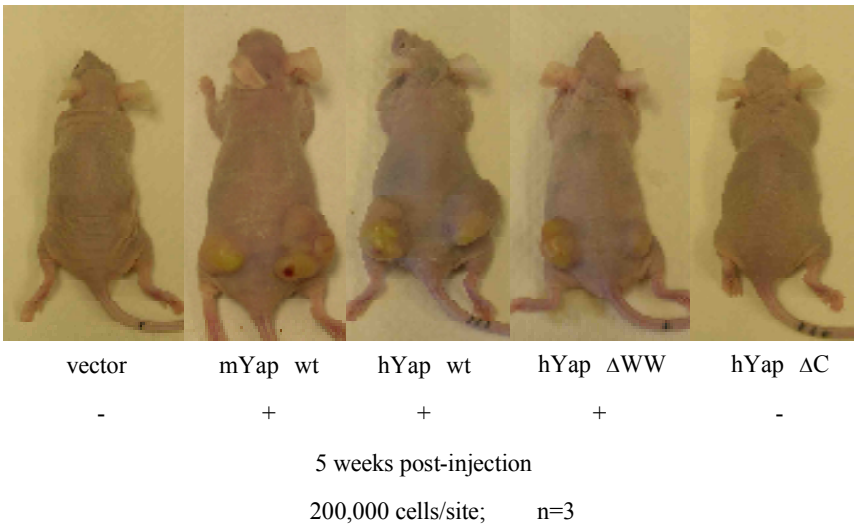

Figure 5: Human Yap1 tumor formation

These results are interesting in light of what we know so far about the structure and function of the Yap protein. If Yap initiates tumor formation through its activity as a transcriptional coactivator, then it makes sense that the ΔC mutant was not only unable to induce tumor formation *in vivo*, but possibly also hampered the ability of endogenous wild-type Yap to promote normal cell cycle progression *in vitro*.

I was concerned, however, that the loss of tumorigenic ability from the ΔC mutant was due to the loss of a TWL-specific function, unrelated to transcription. The TWL motif allows Yap to interact with PDZ domain-containing proteins at the plasma membrane, effectively localizing the Yap protein to the cytosol and allowing to potentially act as a transducer of external signals. I was concerned that the ΔC mutant had lost its oncogenicity not because of a loss of transcriptional function, but because the mutant was no longer able to transduce those signals.

I therefore considered and proposed the following additional experiments to fulfill Aim I:

A. TWL mutant: create by site-directed mutagenesis; test ability to form tumors in nude mice.

B. Establish transcription as the primary means by which Yap1 mediates tumorigenesis.

1. Test cyclin E luciferase activity with wild-type and ΔC mutant Yap.
2. Confirm ability of ΔC mutant Yap to compete with wild-type Yap to bind and transactivate the cyclin E promoter.

The additional site-specific TWL mutant would allow me to dissect the potential roles of signal transduction and transcription from each other and determine whether one specifically allowed Yap to initiate tumorigenesis.

I also sought to compare the ability of wild-type Yap versus delta-C mutant to bind the cyclin E promoter. Since cyclin E gene expression was shown to be upregulated by Yap through our microarray analysis, I asked whether increasing expression of the delta-C mutant could compete with wild-type Yap and abrogate cyclin E gene induction. To this end, I obtained a human cyclin E luciferase reporter and tested the ability of human Yap overexpression to induce luciferase expression. I transfected constant amounts of this reporter plus a constitutive Renilla luciferase control along with varying amounts of MSCV-puro empty vector, MSCV-wild-type Yap and MSCV- delta C constructs.

The assay did not reveal a linear relationship between amount of wild-type Yap transfected and level of luciferase activity compared to cells infected with MSCV-puro alone. The results from this experiment were therefore inconclusive. To resolve this question, I would propose repeating the luciferase assay with another set of genes, including a different cyclin E construct, a DIAP1 promoter, and other genes found highly upregulated by Yap in our microarray data set.

Aim II. Identification of Yap1 downstream effectors

The goal for Aim II was to identify genes positively regulated by Yap that were necessary for Yap to promote an oncogenic effect. To that end, I proposed taking the following approaches:

A. Comparison of genes upregulated by Yap1 in 2 cell types.
    1. Repeat microarray expression analysis of cells with or without Yap1
        - use NIH 3t3 fibroblasts and shp16/p19 immortalized hepatoblasts
        - identify genes that are upregulated by Yap1 in both cell types
    2. Confirm upregulation by Western blot or Q-PCR.

B. Comparison of microRNAs upregulated by Yap1 in 4 cell types
    1. Repeat microRNA microarray analysis for cells with or without Yap1
        - mouse cells: NIH 3t3 fibroblasts and shp16/p19 immortalized hepatoblasts
        - human cells: HepG2 (p53wt) and Hep3B (p53-/-) HCC cells.
    2. Confirm upregulation by Northern blot

C. Test oncogenicity of identified targets
    1. Clone identified downstream microRNAs into MSCV vector and overexpress them.
    2. Test with in vitro colony formation assays and in vivo tumor formation in nude mice

Since it had been recently reported that the microRNA *bantam* plays an essential role in the overproliferation and tumor-like activity induced by Yorkie, the Drosophila homolog of mammalian Yap, my first priority was to pursue Aim IIB, identification of microRNAs whose expression could be upregulated by Yap. I hoped to identify a mammalian counterpart to *bantam* that played a similar essential role in the oncogenicity of mammalian Yap.

II-1. MicroRNA Microarray I.

I harvested total RNA from NIH 3t3 fibroblast cells that been stably infected with MSCV vector or wild-type mouse MSCV-Yap constructs previously described. The cells were infected over a period of 36 hours and then cultured in virus-free media for 12 hours. To ensure a pure population of cells, I then selected the infected cells with puromycin for 2 days and then

recovered the samples in puromycin-free media for 12 hours before harvesting the RNA. The RNA was thus harvested approximately 4 days after the first infection. While the harvested RNA was not enriched for small RNAs prior to microarray hybridization, I used the Ambion miRVana miRNA isolation kit (catalog #AM1560) to minimize the loss of small RNAs during harvesting.

We attempted to hybridize the total RNAs to both the Combimatrix Microarray and the Invitrogen N-Code Microarray Version 2. Both platforms contained probes for microRNAs listed in Sanger database version 9. The Combimatrix arrays were species specific, and we attempted to hybridize the NIH 3t3 samples to the mouse-specific array. The Combimatrix probe list excludes hairpin sequences, and probes are designed to be antisense to mature microRNAs. For each probe, a negative control containing 2 mismatches ("2mut") is included to ensure specificity of the signal. The Invitrogen N-Code Version 2 microarray is a multi-species array containing probes for human, rat, mouse, Drosophila, C. elegans and zebrafish microRNAs listed in the Sanger database. The Invitrogen probes are also antisense to the mature microRNAs, but designed in tandem sequences (approximately double the length of the Combimatrix probes). For each probe, the Invitrogen array contains four negative controls: 1-mismatch ("mut1"), 2-mismatch ("mut2"), reverse complement, and shuffled sequence probes.

We received results first with the Invitrogen N-Code Array. Scoring the genes for highest expression, we identified a list of top candidates (see Table 2).

Table 2: INVITROGEN Ncode MicroRNA Microarray #1

name	M value	A value	p-value
hsa-let-7d_mmu-let-7d_rno-let-7d	1.209218466	8.429519285	0.040652645
mmu-miR-300_rno-miR-300	1.46966157	5.440632727	0.08883108
dre-miR-16a	1.818329984	4.445537387	0.070489613
Dye Marker	1.897085711	7.003151045	0.067717284
dre-miR-17a	2.007483997	3.7456597	0.053563107
mmu-miR-670	2.123900592	3.536218198	0.07442276
mmu-miR-489	2.350912762	6.475881128	0.051250771
hsa-miR-16_mmu-miR-16_rno-miR-16	2.431607443	3.999521032	0.06518515
dre-miR-10d	2.716993189	3.140723668	0.099045113
hsa-miR-18a_mmu-miR-18_rno-miR-18_dre-miR-18a	2.838270012	3.350768359	0.089396596
hsa-miR-518d	2.875142269	5.039594841	0.086681044
dme-miR-87	2.886815072	2.519919565	0.085841331
hsa-miR-17-5p_mmu-miR-17-5p_rno-miR-17	**2.918802546**	**6.598085204**	**0.008770827**
dre-miR-27d	**3.15123766**	**4.339383951**	**0.069132038**
hsa-miR-29b_mmu-miR-29b_rno-miR-29b	3.162898813	5.802509122	0.068487478
dre-miR-27e	3.177928401	2.697139498	0.06766711
Dye Marker	3.311772777	9.232654689	0.025818579
dre-miR-27d	**3.328472412**	**4.444378792**	**0.060057067**
Blank_P24	3.533447389	3.808339217	0.051261661
1570-mut1-has-mir-152	3.542624644	2.72658143	0.050905096
dre-miR-16b	**3.620024003**	**3.305181494**	**0.04801272**
dre-miR-29b	3.72456536	3.070279664	0.044411773
dre-let-7g	3.962581815	2.905089999	0.037353774
hsa-miR-18a_mmu-miR-18_rno-miR-18_dre-miR-18a	4.053427481	4.216451632	0.035021766
dre-miR-153c	4.7457885	3.110806774	0.022022246
dre-miR-27d	**4.936924989**	**2.425407949**	**0.019531198**
hsa-miR-127_rno-miR-127	**6.223373482**	**4.041858602**	**0.009412026**

We chose several candidates to validate by Northern blot. We especially wanted to investigate the upregulation of the miR-17-5p and miR-18a microRNAs, mature transcripts from the miR17-92 locus, as reported by the NCode study. Recent reports had identified the miR-17-92 locus as being an oncogenic locus for B-cell lymphomas driven by the *c-myc* oncogene (He et al., 2005), and we wanted to investigate this for hepatocellular carcinomas.

Initial studies by Northern blot did not reveal a significant increase in miR17-5p and miR18a levels in Yap-overexpressing cells. I therefore attempted to validate the microarray result using two additional approaches: Taqman QPCR and luciferase assay. The Taqman QPCR attempts were also inconclusive. For the luciferase assay, I used the miR-17-92 luciferase reporter described by Woods et al., 2006 (pro1353, the 1353 bp prior to the start of miR17-92 transcription). A NIH 3t3 cell line stably infected with a tet-inducible Yap construct (see Aim II-2) was transiently transfected with pro1353 and a Renilla control. Yap expression was induced with doxycycline in half the cells and luciferase activity measured 24 hours after induction. The luciferase assay results revealed that there were no significant differences in activity at the miR17-92 promoter between induced and uninduced cells (see Table 3).

Table 3: miR17-92 Luciferase Assay

sample	pro1353	renilla	ratio	average
control	2544	19092	0.133272	0.146813
control	3108	18665	0.166513	
control	2476	17605	0.140655	
yap	4866	36647	0.132785	0.145757
yap	5389	33065	0.162988	
yap	5778	40833	0.141498	

II-2. Creation of tet-inducible Yap constructs.

Since we were unsuccessful in our attempts to validate the initial microarray results, we became concerned that the microRNAs that looked most highly expressed by Yap in the microarray were not direct targets of Yap, but genes secondarily upregulated by mediators of Yap overexpression. I designed a tet-on system to control Yap protein expression and study gene upregulation at time points shortly after Yap overexpression was induced. This system depended on two constructs, a neomycin-selectable MSCV-rtTA3 construct that was first introduced into 4 cell lines (mouse NIH3t3 fibroblasts, mouse shp16/p19 hepatocytes, human HUH7, and human HepG2) and selected with G418 for 2 weeks. Next, I introduced a puromycin-selectable TRE-Yap construct with either mouse or human wild-type Yap, and selected the cells for 2 days.

I first tested NIH 3t3 cells harboring TRE-mouse Yap for induction with 1 ug/mL doxycycline and found that even at 8 hours post induction, Yap was strongly induced (see Western blot below). However, even at high levels of Yap overexpression, I saw no difference in cyclin E expression between induced and uninduced samples. This is in apparent contrast to our initial expression microarray results. However, it maybe worth noting that the original expression microarray analyzed tumor samples, and this Western blot correlates to a short time frame *in vitro*.

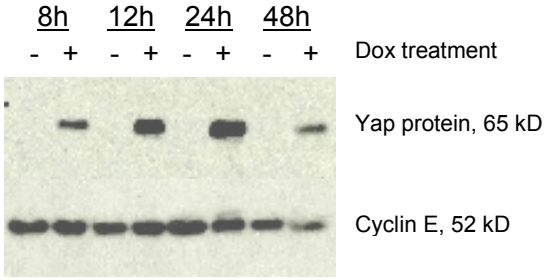

Figure 6: Doxycycline induction of Yap1 protein expression

II-3. Second microRNA microarray experiment

Using NIH 3t3 cells stably infected with the tet-inducible Yap system, I collected total RNA after 12 and 24 hours of doxycyline induction and repeated the Invitrogen N-Code microarrays. This time, I compared the microarray data between the two time points and found that they were strongly correlated. Table 4 demonstrates the correlation between the two data points, and lists the microRNAs that were found to express at the highest levels. The next step would be to validate these targets by Northern blot and Taqman QPCR.

Table 4: Comparison of microRNAs upregulated after 12 hours or 24 hours of doxycycline induced Yap1 overexpression

mir	exp24_med	exp12hr_med
Mmu-miR-411	-7.8977122	-7.944233744
hsa-miR-520h	-7.7979727	-7.948685131
cel-miR-49	-7.5120672	-7.496015552
Mmu-miR-700	-7.1760264	-6.950391466
dre-miR-462	-7.0751376	-7.266721961
1548-rev-cel-mir-244	-7.0527919	-7.130217559
hsa-miR-487b	-6.9645061	-7.26736782
hsa-miR-524*	-6.9080998	-7.050064343
Mmu-miR-487b_rno-miR-487b	-6.8915783	-7.19482421
rno-miR-382*	-6.6817324	-6.631875748
Dme-miR-286	-6.253013	-6.245700901
Mmu-miR-466	-6.1472627	-5.877787609
hsa-miR-197	-5.989366	-5.80338472
rno-miR-342_mmu-miR-342_hsa-miR-342	-5.6062647	-5.641221084
hsa-miR-668_mmu-miR-668	-5.5461323	-5.379227538
dre-miR-219	-5.4296972	-4.955750149
Dye Marker	-5.3069761	-5.106712524
hsa-miR-219_mmu-miR-219_rno-miR-219	-5.1248148	-5.195282844
Dme-miR-305	-4.8691163	-5.189564693
dre-miR-220	-4.8691106	-4.768515977
hsa-miR-378_mmu-miR-378_rno-miR-378	-4.8174432	-5.580747566
Dme-miR-219	-4.7416491	-4.467037568
hsa-miR-224_mmu-miR-224_rno-miR-224	-4.4502316	-5.163974387
Mmu-miR-698	-4.3900719	-5.046333349
cel-miR-53	-4.3206373	-5.07159236
1568-rev-mmu-mir-325	-4.2632689	-3.924655552
Mmu-miR-674*	-4.1586662	-4.30031701
hsa-miR-595	-4.1250088	-4.360757502
Mmu-miR-676*	-4.0640233	-4.104371514
rno-miR-376b	-3.9165574	-3.953287378
Mmu-miR-467a	-3.8735553	-3.708598145
Mmu-miR-467b	-3.5693295	-3.244553572
Dme-miR-287	-3.5581923	-3.929057105
1552-rev-mmu-mir-292	-3.4869705	-3.435108019

Aim III. Targeted Therapy for 11q22 tumors

My purpose in studying how Yap mediates tumorigenesis was to identify new therapeutic targets specific to tumors containing the 11q22 amplicon. We had previously shown that murine tumors driven by the 9qA1 amplicon develop more slowly when Yap is depleted through short hairpin knockdown, even though the depletion was incomplete (Zender et al., 2006). If these tumors indeed require Yap overexpression to maintain malignancy, then an effective strategy for treating these tumors could involve targeting Yap or its downstream effectors.

The goal for Aim III was to take the genes validated in Aim II as essential effectors of Yap oncogenicity, and use that information to design therapies targeted against tumors driven by the 11q22 amplicon. I planned to divide the work as follows:

A. Establish requirement for Yap1 in 11q22 tumors
   1. In vitro: Test hairpins against mouse and human Yap1 in 9qA1 (mouse) and 11q22 (human) tumors; measure proliferation.
   2. In vivo: Infect 11q22+/- tumors with rtTA (tet-on) and TRE-shYap plasmids. Inject cells subcutaneously into nude mice and allow tumors to develop. Feed mice doxycycline and measure effect on tumor burden.

B. Test effect of Yap1 effectors in 11q22 tumors
   - same experiments as with Yap1, but with hairpins/antagomirs against validated targets.
   - compare with shRNA against Yap1.

Fulfillment of Aim III depended on the success of Aim II.

III-1. Characterization of human 11q22 amplicon-positive tumors.

As preliminary work before beginning Aim III, I examined human tumor lines that contain the 11q22 amplicon for the expression of Yap and its downstream targets. The first set of cell lines I received were non-small cell lung carcinoma lines H2086 (11q22 positive), SKLU-1 and EPLC (11q22 negative). By Western blot, I found that H2086 expressed extremely high levels of Yap and cyclin E, a gene we had identified in our microarray as a target upregulated by Yap (Figure 5). EPLC and SKLU-1, on the other hand, expressed low levels of Yap in comparison. Our microarray data also show that Yap increases the expression of upstream signaling molecules in the MAPK pathway, such as platelet-derived growth factor receptor (PDGF-R). Interestingly, H2086 cells show high Erk phosphorylation, an indicator of MAPK pathway activation, relative to EPLC and SKLU-1. Ponceau staining of the membrane showed equal loading of the lanes.

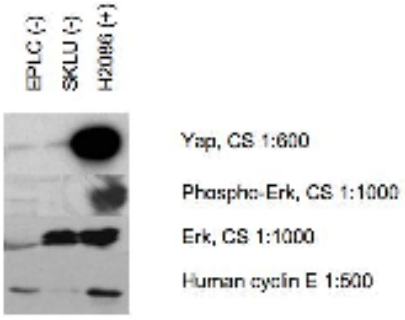

Figure 7: Characterization of human 11q22+ tumors

Based on the similarity between these characteristics of the H2086 cell line and our results using murine tumors harboring the syntenic 9qA1 amplicon, I decided I could use the H2086 cell line as a human counterpart to experiments using murine tumors. I designed hairpins against human Yap and in two separate experiments, tried to evaluate their efficacy in the H2086 cell line by Western blot. However, while MSCV vector-infected H2086 cells grew, the hairpin-

infected cells died after puromycin selection and I could not obtain samples for the Western. At this point I do not know whether this result was due to experimental error or because the H2086 cells are sensitized to a decrease in Yap protein levels. The best way to resolve this issue would be to repeat the hairpin evaluation using NIH 3t3 cells harboring tet-inducible human Yap, which are not sensitized to Yap overexpression, and evaluating knockdown in dox-induced cells.

Summary

The goal of my thesis work was to identify the downstream effectors of the Yap1 oncoprotein that contributed significantly, if not primarily, to the effects of Yap1 overexpression seen in tumors harboring the 11q22 amplicon. Based on my analysis of the structure and function of Yap, as well as recent work by others describing the requirement for nuclear localization in Yap-mediated tumorigenesis (Dong et al., Cell 2007), it seems that Yap1 can initiate and maintain tumorigenesis at high expression levels when an overabundance of Yap1 results in a high level of transcriptional activity. Loss of the C-terminal transactivation domain or loss of nuclear localization both result in loss of Yap1 oncogenic activity.

Because I believe that the oncogenicity of Yap depends on its role as a transcriptional coactivator, I have sought to identify the gene or genes transactivated by Yap that are essential for Yap to promote tumorigenesis. I focused on identifying microRNAs upregulated by Yap and have identified a list of candidates that require validation by Northern blot and QPCR. My initial microarray results did not validate by Northern blot or luciferase assay, possibly because my experimental conditions added factors that obscured the primary effects of Yap overexpression. Possible causes of interference include a) using puromycin to select for Yap expression, which is known to stress even puromycin-resistant cells and b) harvesting RNA at a late time point that revealed secondary, not primary effects. To minimize these potential causes of interference, I created a tet-inducible Yap system in both mouse and human cell lines, and harvested RNA from earlier time points.

I also sought to collect human tumor lines harboring the 11q22 amplicon and examine their dependence on Yap overexpression. I expected to see the tumor lines respond to shRNA depletion of Yap1 by growing more slowly, and I hoped to identify downstream effectors of Yap1 that could fully or partially recapitulate that effect. I began by attempting to evaluate the potency of several hairpins against human Yap by infecting the Yap-overexpressing H2086 non-small-cell lung carcinoma tumor line, but hairpin-infected cells did not grow. It is possible that H2086 cells are sensitized to Yap depletion. If this is the case, then hairpins should first be evaluated in non-sensitized cells, such as NIH 3t3 cells harboring tet-inducible human Yap. Second, hairpins or antagomirs against targets identified in Aim II should be evaluated for their ability to recapitulate the lethality of the shRNA depletion of Yap in H2086 cells. Ideally, this experiment would be repeated in other 11q22 amplicon-containing tumors, both human and murine, to identify common mammalian effector of Yap that are required for tumorigenesis.

References

Bashyam M.D., R. Bair, et al. (2005). "Array-based comparative genomic hybridization identifies localized DNA amplifications and homozygous deletions in pancreatic cancer." Neoplasia **7**(6):556-62)

Basu, S., N.F. Totty, et al. (2003). "Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis." Mol. Cell **11**:11-23.

Bork, P. and M. Sudol (1994). "The WW domain: a signaling site in dystrophin?" Trends Biochem. Sci. **19**:531-533).

Dai Z., W.G. Zhu, et al. (2003). "A comprehensive search for DNA amplification in lung cancer identifies inhibitors of apoptosis cIAP1 and cIAP2 as candidate oncogenes." Hum. Mol. Genet. **12**(7):791-801.

Dickins R.A., M.T. Hemann, et al. (2005). "Probing tumor phenotypes using stable and regulated synthetic microRNA precursors." Nat. Genet. **37**(11):1289-95.

Dong J., G. Feldmann, et al. (2007). "Elucidation of a universal size-control mechanism from *Drosophila* to mammals." Cell, 130:1120-1133.

Farazi P.A. and R.A. DePinho (2006). "Hepatocellular carcinoma pathogenesis: from genes to environment." Nat. Rev. Cancer **6**(9):674-87.

Garnier, L.., J.W. Wills, et al. (1996). "WW domains and retrovirus budding." Nature **381** (6585):744-5.

Gudas J.M., M. Payton, et al. (1999). "Cyclin E2, a novel G1 cyclin that binds cdk2 and is aberrantly expressed in human cancers." Mol. Cell Biol. **19:**612-622.

He L., Thompson J.M.,et al. (2005) "A microRNA polycistron as a potential human oncogene." Nature. **435**:828-33.

Huang, J., W. Shian, et al. (2005). "The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the *Drosophila* homolog of Yap." Cell **122**:421-434

Imoto I., Z.Q. Yang, et al. (2001). "Identification of cIAP1 as a candidate target gene within an amplicon at 11q22 in esophageal squamous cell carcinomas." Cancer Res. **61**(18):6629-34.

Kanai F., P.A. Marignani, et al. (2000). "TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins." EMBO J. **19** (24): 6778-6791.

Lang, L. (2008). "FDA approves sorafenib for patients with inoperable liver cancer." Gastroenterology. **134**(2):379

Levy, D., Y. Adamovich., et al. (2006). "The Yes-associated protein 1 stabilizes p73 by preventing Itch-mediated ubiquitination of p73." Cell Death and Diff. 1-9.

Lowe, S.W., H.E. Ruley, et al. (1993). "p53-dependent apoptosis modulates the cytotoxicity of anticancer agents." Cell **74**(6):957-67.

Mohler P.J., S.M. Kreda, et al. (1999). "Yes-associated protein 65 localizes p62/c-Yes to the apical compartment of airway epithelia by association with EBP50." J. Cell. Biol. **147**(4):879-890.

Morin-Kenisicki, E.M., B.N. Boone, et al. (2006). "Defects in yolk sac vasculogenesis, chorioallantoic fusion, and embryonic axis elongation in mice with targeted disruption of Yap65." Mol. Cell. Biol. **26**(1):77-87.

Samuelson A.W., M. Narita, et al. (2005). "p400 is required for E1A to promote apoptosis." J. Biol. Chem. **280**(23):21915-21923.

Snijders A.M., B.L. Schmidt, et al. (2005). "Rare amplicons implicate frequent deregulation of cell fate specification pathways in oral squamous cell carcinoma." Oncogene **24** (26):4232-42.

Strano, S., E. Munarriz, et al. (2001). "Physical interaction with Yes-associated protein enhances p73 transcriptional activity." J. Biol. Chem. **276** (18):15164-15173.

Strano, S., O. Monti, et al. (2005). "The transcriptional coactivator Yes-associated protein drives p73 gene-target specificity in response to DNA damage." Mol. Cell **18**:447-459.

Sudol, M. (1994) "Yes-associated protein (Yap65) is a proline-rich phosphoprotein that binds to the SH3 domain of the Yes proto-oncogene product." Oncogene **9**: 2145-2152.

Sudol, M., P. Bork, et al. (1995). "Characterization of the mammalian YAP gene and its role in defining a novel protein module, the WW domain." J Biol Chem. **270**(24):14733-41.

Thompson, B.J. and S.M. Cohen (2006). "The Hippo pathway regulates the *bantam* microRNA to control cell proliferation and apoptosis in *Drosophila.*" Cell **126**:767-774

Yagi, R., L. Chen, et al. (1999). "A WW domain-containing Yes-associated protein (YAP) is a novel transcriptional co-activator." EMBO J. **18**(9):2551-62.

Weinstein I.B. (2002). "Addiction to oncogenes – the Achilles heel of cancer." Science **297**:63-64.

Woods, K., et al. (2006). "Direct regulation of an oncogenic microRNA cluster by E2F transcription factors."
J. Biol. Chem. 282:2130-2134.

Xue W., L. Zender, et al. (2007). "Senescence and tumor clearance is triggered by p53 restoration in murine liver
carcinomas." Nature **445** (7128):656-60.

Zender, L., M.S. Spector, et al. (2006). "Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach." Cell **125**: 1253-1267.