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In vivo activation of Wnt/β-catenin signaling to model Schwann cell tumors in mice

Introduction

While Wnt/β-catenin signaling has been demonstrated to be involved in a variety of cancers, such as lung, liver and brain tumors, it was only recently that our lab implicated this pathway in the development of Schwann cell tumors, including benign tumors known as neurofibromas, and malignant peripheral nerve sheath tumors (MPNSTs)—aggressive soft tissue sarcomas that originate in Schwan cells or Schwann cell precursors. (Watson et al., Cancer Discovery, 2013; Carrol and Ratner, Glia, 2008; MacDonald et al., Dev Cell, 2009). Approximately half of MPNSTs are developed by patients with Neurofibromatosis Type 1 Syndrome (NF1), a genetic disease characterized by a heterozygous loss of the Neurofibromin 1 gene (*NF1*), and half occur spontaneously (Evans et al, J Med Genet, 2002). In its normal role as a Ras GTPase-activating protein, *NF1* functions as a tumor suppressor gene by negatively regulating Ras (Xu et al, Cell, 1990). It is known that in NF1 patients, loss of the second copy of the *NF1* gene results in the development of neurofibromas, and 10% of these will incur further genetic changes that result in the transformation to MPNSTs (Evans et al, J Med Genet, 2002; Katz et al., Expert Rev Mol Med, 2009). Current treatments of MPNSTs are far from effective, with 5-year survival rates currently below 40%; therefore a better understanding of the genetic mechanisms by which these tumors form and progress is essential to developing better therapies (Kolberg, Neuro Oncol., 2013).

Research in the Largaespada lab, utilizing a *Sleeping Beauty* forward genetic screen, identified multiple genes implicating canonical Wnt signaling in the development of MPNSTs (Rahrmann et al, Nature Genetics, 2013). Several mouse models for MPNSTs also demonstrate activation of this pathway (Watson et al, Cancer Discovery, 2013). In addition, quantitative real-time PCR (QPCR), gene expression microarray, and tissue microarray analyses have confirmed activation of this pathway in many or most human neurofibromas and MPNST samples (Watson et al, Cancer Discovery, 2013). *In vitro* studies have shown that activation of Wnt signaling alone was able to produce oncogenic phenotypes in immortalized human Schwann cells, and down-regulation of the pathway in human MPNST cells was able to mitigate the oncogenic properties in these cells. In immortalized human Schwann cell lines, activating Wnt/β-catenin signaling by multiple mechanisms, including overexpression of activated β-catenin, and knockdown of β-catenin destruction member components, was sufficient to increase cellular proliferation and anchorage-independent growth (Watson et al, Cancer Discovery, 2013). Using shRNA constructs targeted for β-catenin, we observed diminished Wnt signaling, as well as reduced proliferation, anchorage-independent growth, and xenograft tumor formation in both NF1-associated and sporadic MPNST cell lines (Watson et al, Cancer Discovery, 2013). While these studies point to the importance of Wnt/β-catenin signaling *in vitro*, they do not demonstrate what effect overexpression of β-catenin and activation of the Wnt pathway in Schwann cells has in the context of an *in vivo* mouse model. It was our hypothesis that by expressing an activated

form of β-catenin in the Schwann cells of mice, Wnt signaling would be activated, and nerve hyperplasia and Schwann cell tumors would develop. In addition, as it has previously been shown in an *in vivo* mouse model that bi-allelic loss of *Nf1* in the Schwann cells of adult mice leads to the development of neurofibromas, we predicted that expression of activated β-catenin with this background would act cooperatively in the development of Schwann cell tumors, resulting in decreased tumor latency, increased tumor burden, and higher tumor grade (Mayes et al, Cancer Research, 2011; Wu et al, Cancer Cell, 2008).

Methods

In order to investigate the role of Wnt signaling in the development of MPNSTs *in vivo*, we utilized a mouse model expressing an activated form of β-catenin in Schwann cells. Mutations that stabilize β-catenin and prevent its degradation are found in a variety of human cancers, allowing activation of Wnt signaling (Harada et al, EMBO, 1999). Taking advantage of this, we implemented a Cre-Lox system in which Exon 3 of *β-catenin*, which contains a critical site of phosphorylation, is between *lox*P sequences (*Ctnnb1*$^{lox(ex3)}$), and its removal results in a constitutively active form of β-catenin (Harada et al, EMBO, 1999). In a separate mouse strain, the desert hedgehog (*Dhh)* regulatory sequence was used to control expression of Cre Recombinase in Schwann cells and Schwann cell precursors, the cell of origin for neurofibromas and MPNSTs (Keng et al, Sarcoma, 2012; Carroll and Ratner, Glia, 2008; ; Wu et al, Cancer Cell, 2008).When *Ctnnb1*$^{lox(ex3)}$ mice and *Dhh-Cre* are crossed, *Dhh-Cre*; *Ctnnb1*$^{lox(ex3)}$ mice will be produced that express the activated form of β-catenin in the Schwann cells and Schwann cell precursors. As activation of the Wnt pathway has been shown to induce an oncogenic phenotype *in vitro*, we hypothesized that activation of this pathway through the expression of an activated form of β-catenin would have the a similar effect in an *in vivo* mouse model, leading to nerve hyperplasia, and the formation of peripheral nervous system tumors that model Schwann cell tumors seen in human patients. In addition, to determine if activation of Wnt signaling cooperates with loss of *Nf1* in mice for Schwann cell tumorigenesis, we concurrently crossed in an *Nf1* floxed allele to our *Dhh-Cre*; *Ctnnb1*$^{lox(ex3)}$ mice (Wu et al, Cancer Cell, 2008) . This cross would result in mice of three possible genotypes: *Dhh-Cre*; *Ctnnb1*$^{lox(ex3)}$, *Dhh-Cre*; *Nf1*$^{flox/+}$; *Ctnnb1*$^{lox(ex3)}$, and *Dhh-Cre*; *Nf1*$^{flox/flox}$; *Ctnnb1*$^{lox(ex3)}$. As it is known that loss of *NF1* alone is insufficient for malignant tumor formation and other genetic changes are necessary for this to occur, characterization of these progeny would give us insight into the effect of Wnt activation on MPNST development in NF1 patients (Bajenaru et al, Mol Cell Biol, 2002; Wu et al, Cancer Cell, 2008).

DNA was isolated from tail clippings using proteinase K treatment, phenyl chloroform extraction, and ethanol precipitation, and was dissolved in sterile TE [10 mmol/L tris-HCl (pH7.5), 1 mmol/L EDTA (pH 8)]. For PCR genotyping, three primers were used to distinguish between wild-type (WT) *Ctnnb1* and *Ctnnb1*$^{lox(ex3}$: 5' GGACAATGGCTACTCAAGGTTTGTG 3', 5' CTAAGCTTGGCTGGACGTAAACTC 3', and 5'ACGTGT GGCAAGTTCCGCGTCATCC 3' (Jackson Laboratories). Primers for the *Nf1* floxed allele were WT forward 5' CTTCAGACTGATT GTTGTAACTGA 3', WT reverse 5' ACCTCTCTAGCCTCAGGAATGA 3', and floxed

reverse 5' TGATTCCC ACTTTGTGGTTCTAAG; and for *Dhh-Cre* were forward 5' CTGGCCTGGTCTGGACACAGTGCC 3' , and reverse 5' CAGGGTCCGCTCGGGCATAC 3' (Keng et al, Cancer Res., 2012). PCR products were separated on 2% agarose gel and presence or absence of amplicons used to determine genotype. Mice of desired genotypes were weaned, and subsequently monitored daily for signs of overall health, including weight, mobility, and coordination. When moribund, necropsies were performed in which any tumors were characterized by number, size, and grade; and nerves were observed for hyperplasia and tumor formation. All abnormal nerves were assessed for tumor grade using well established immunohistochemistry and staining methods. Specifically, sections of formalin fixed tissue were stained with hemotoxin and eosin, S100 (a Schwann cell marker), and Ki67 (proliferation marker), and were examined histopathologically for signs of hyperplasia and/or tumor grade (Keng et al, Sarcoma, 2012).

Results

During the extensive breeding process, it became apparent that we were getting far fewer *Dhh*-Cre; *Ctnnb1*$^{lox(ex3)}$ pups than would be expected by Mendelian inheritance of the alleles. It appears as though activation of Wnt signaling in *Dhh* positive cells is semi-lethal, as just four *Dhh-Cre*; *Ctnnb1*$^{lox(ex3)}$ mice were generated, each of which died before necropsies could be performed, living an average 33 days (Figure 1). Ten *Dhh-Cre*; *Nf1*$^{flox/+}$; *Ctnnb1*$^{lox(ex3)}$ mice were generated, living an average of approximately 44 days (Figure 1). Five of these mice died before necropsies were performed, and necropsies of these remaining mice revealed no tumors or enlarged nerves (Figure 2). Also of interest, each of these five mice was revealed to have an enlarged heart, and several displayed discoloration of the lungs and/or intestines. The four *Dhh-Cre*; *Nf1*$^{flox/flox}$; *Ctnnb1*$^{lox(ex3)}$ mice generated lived an average of ~48 days, two of which died prior to necropsy, and examination of the remaining two mice revealed no tumors and no enlarged nerves (Figure 1, 2). While tumor formation or significant nerve hyperplasia was not observed in any of the mice generated, this may be at least partially attributed to the drastically reduced life span that was characteristic of this mouse model. In comparison to *Dhh-Cre; Nf1*$^{flox/+}$ and *Dhh-Cre; Nf1*$^{flox/flox}$ mice, mice expressing activated β-catenin—regardless if *Nf1* alleles were wild-type or floxed—had substantially diminished life-spans, the longest of which was just 77 days. Analysis using two-tailed t-tests revealed difference in length of life between *Dhh-Cre; Nf1*$^{flox/+}$ or *Dhh-Cre; Nf1*$^{flox/flox}$ and each cohort expressing activated β-catenin to be very significant (p-value <<0.0001) (Figure 1). However, differences in length of life were not statistically significant between the three cohorts expressing activated β-catenin, demonstrating that activated β-catenin expression is largely responsible for the diminished viability of these mice (Figure 1).

Discussion and Future Direction

In addition to conclusions regarding abnormal Wnt/β-catenin signaling, we also see the importance of Wnt/β-catenin signaling in normal development. Normally, this signaling pathway is strictly regulated, and in the absence of Wnt signaling, β-catenin is phosphorylated and tagged for destruction by the β-catenin destruction

complex. However, the activated form of β-catenin utilized in this mouse model cannot be phosphorylated, and even in the absence of extracellular Wnt signaling can accumulate in the cytoplasm, enter the nucleus, complex with members of the Tcf/Lef family, and act as a transcriptional activator of Wnt target genes, including cell proliferation activators such as c-Myc and cyclin D1 (Barker, Methods Mol Biol, 2008). It is clear for this mouse model that when Schwann cells constitutively receive proliferative signals in the absence of stimulatory signaling, the abnormal signaling and proliferation that results has disastrous consequences for development; evidenced in the small cohorts generated, diminished length of life, and phenotypic abnormalities, including discoloration of organs, and enlargement of the heart. Given the pervasiveness of this last phenotype, it is likely that expression of activated β-catenin in Schwann cells results in abnormal heart development, contributing greatly to the diminished viability of this model. Schwann cells are known to serve an important role in angiogenesis and neural remodeling in cardiac ganglia through their secretion of cytokines such as vascular endothelial growth factor (VEGF), and it is likely that the expression of activated β-catenin alters this signaling in a manner which often proves lethal (Wang et al, Mol Cell Biochem, 2012). VEGF is a pleiotropic factor in the developing embryonic heart, regulating vasculogenesis and angiogenesis, as well as survival in endothelial cells (Armstrong and Bischoff, Heart Valve Development, 2004). As activated β-catenin expression has been shown to up-regulate VEGF-A mRNA and protein levels by three fold in normal colon epithelial cells, it is plausible that this mechanism is also acting in Schwann cells, resulting in the characteristic enlarge heart phenotype we observed, as well as the semi-lethal consequence of activated β-catenin expression (Easwaran et al, Cancer Res, 2003).

Apart from demonstrating important consequence of activated β-catenin expression in the Schwann cells of developing mice, these experiments provide a foundation on which to pursue the full characterization of a related model—one which implements an inducible Cre-Lox recombination system. A fusion between Cre recombinase and a mutated ligand-binding domain of the human estrogen receptor (ER) yields a tamoxifen dependent Cre recombinase, Cre-ER^T (Feil et al, Handb Exp Pharmacol, 2007). In the absence of tamoxifen, Cre-ER^T is bound to heat shock protein-90 in the cytoplasm and is therefore inactive; however, upon treatment with tamoxifen, Cre-ER^T binds tamoxifen, translocates to the nucleus, and engages target DNA to induce recombination (Liu et al, PLos One. 2010). In order to cause recombination specifically in Schwann cells, regulatory sequences of myelin proteolipid protein (Plp)—a component of Schwann cell myelin sheath—has been used to control *Cre-ER^T* transcription (Mallon et al, Neurosci, 2002; Doerflinger et al, genesis, 2002). *Cre-ER^T* under this transcriptional control—designated *Plp-Cre*—has been successfully implemented to induce *Nf1* loss of function in an *in vivo* mouse model, and demonstrated that *Nf1* inactivation in Schwann cells after birth or in adult animals results in enlarged peripheral nerves, and formation of neurofibromas (Mayes et al, Cancer Research, 2011). We will utilize this *Plp-Cre* recombination system to induce the expression of activated β-catenin and the inactivation of *Nf1* expression in adult mice, allowing the developmental consequences encountered with the *Dhh-Cre* system to be avoided. The experimental plan is as follows:

1. *Plp-Cre* mice will be crossed with *Nf1$^{flox/flox}$; Ctnnb1$^{lox(ex3)}$* mice and the resulting offspring will be intercrossed to obtain 30 mice per cohort of *Plp-Cre; Ctnnb1$^{lox(ex3)/+}$, Plp-Cre; Nf1$^{flox/+}$; Ctnnb1$^{lox(ex3)/+}$,* and *PLP-Cre; Nf1^{flox,flox}; Ctnnb1$^{lox(ex3)/+}$*. In addition 15 *PLP-Cre; Nf1$^{lox/flox}$* will be generated. Crosses are currently in progress for this model, and we will shortly begin tamoxifen injections (Table 1).
2. Mice will be aged to 60 days at which point they will be injected twice a day for three consecutive days with 1mg tamoxifen dissolved in 100 μL 9:1 sunflower seed oil/ethanol to induce recombination (Mayes et al, Cancer Research, 2011). Mice will be monitored daily for signs of overall health, including weight, mobility, and coordination.
3. When mice become moribund, necropsies will be performed in which tumors will be characterized by number, size, and grade; and nerves will be observed for hyperplasia and tumor formation. All abnormal nerves will be assessed for tumor grade using well established immunohistochemistry and staining methods. Specifically, sections of formalin fixed tissue will be stained with hemotoxin and eosin, S100 (a Schwann cell marker), and Ki67 (proliferation marker), and will be examined histopathologically for signs of hyperplasia and/or tumor grade (Keng et al, Sarcoma, 2012).
4. Two methods will be used to validate that Wnt signaling has been activated. Western blot analysis will be used to confirm expression of activated β-catenin, and QPCR will be used to quantify expression of genes activated by Wnt signaling, including *C-Myc (MYC), lymphoid enhancer-binding factor 1 (LEF1), axis inhibitor 2 (AXIN2), CyclinD1 (CCND1),* and *Survivin (BIRC5).* Expression levels will be compared between wild type mice, *Plp-Cre; Nf1$^{flox/flox}$* mice, and the three experimental groups expressing activated β-catenin, as well as in Schwann cells versus other cell types to verify that Wnt signaling is only activated in *Plp-Cre* expressing cells.

Utilization of this strategy will allow us to avoid the disastrous effects activated β-catenin expression poses to normal development, and give us insight into the role of this signaling pathway in Schwann cell tumorigenesis, for both spontaneous cases and those in the context of NF1 patients.

Tables and Figures

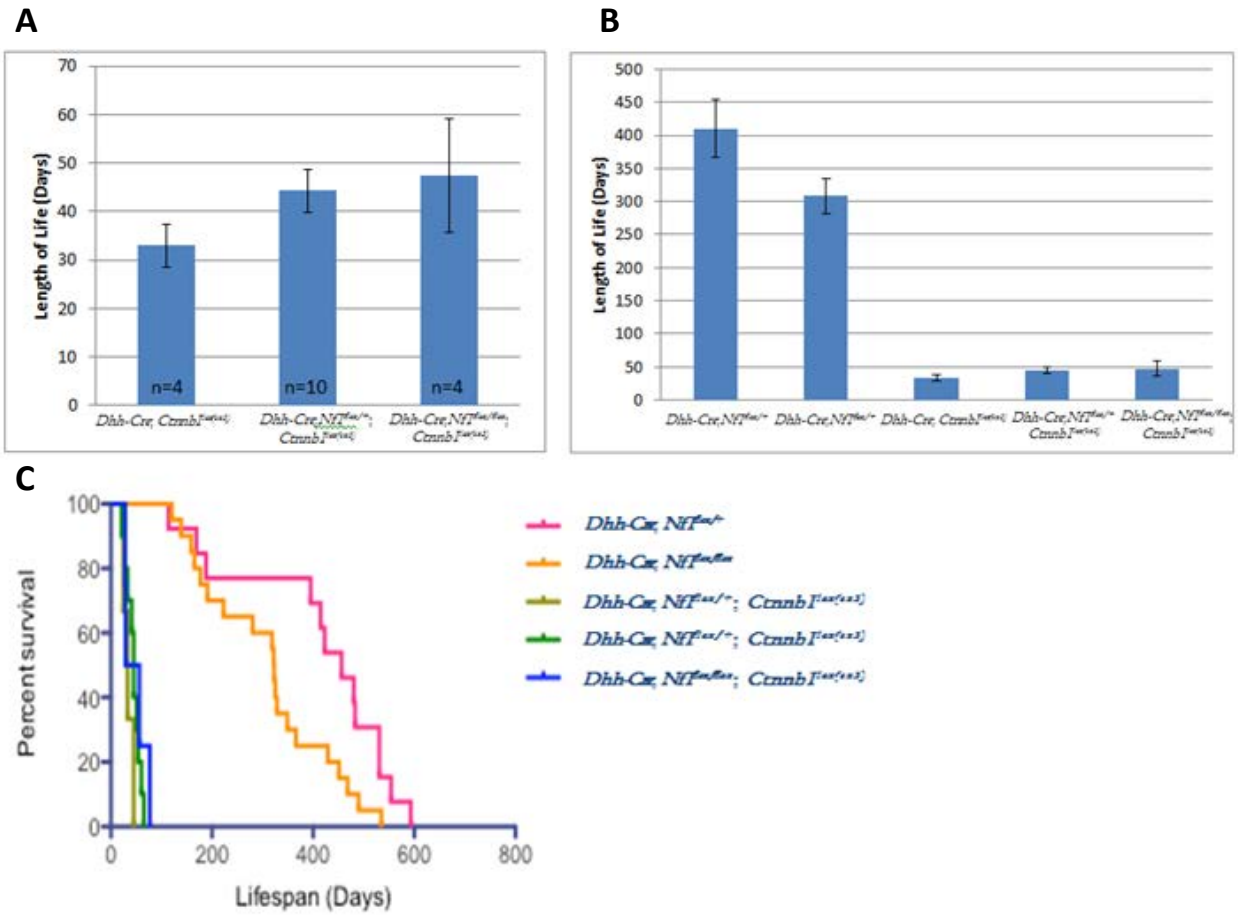

Figure 1

Average length of life for **A)** *Dhh-Cre*; *Ctnnb1*$^{lox(ex3)}$, *Dhh-Cre*; *Nf1*$^{flox/+}$; *Ctnnb1*$^{lox(ex3)}$, and *Dhh-Cre*; *Nf1*$^{flox/flox}$; *Ctnnb1*$^{lox(ex3)}$ mice were not significantly different from one another (p-values>0.05) according to two-tailed t-test. SEM error bars included. **B)** *Dhh-Cre*; *Ctnnb1*$^{lox(ex3)}$, *Dhh-Cre*; *Nf1*$^{flox/+}$; *Ctnnb1*$^{lox(ex3)}$, and *Dhh-Cre*; *Nf1*$^{flox/flox}$; *Ctnnb1*$^{lox(ex3)}$ mice lived significantly shorter than *Dhh-Cre*; *Nf1*$^{flox/+}$ and *Dhh-Cre*; *Nf1*$^{flox/flox}$ (p-values<<0.0001) according to two-tailed t-test. SEM error bars included. **C)** Kaplan Meier graph showing percent survival as a function of time (days) of experimental cohorts in reference to *Dhh-Cre*; *Nf1*$^{flox/+}$ and *Dhh-Cre*; *Nf1*$^{flox/flox}$ mice. Mice were monitored daily for signs of overall health and euthanized when paralyzed, failed to groom, or lost significant weight. Graph is representative of four *Dhh-Cre*; *Ctnnb1*$^{lox(ex3)}$ mice, all of which died before necropsy; ten *Dhh-Cre*; *Nf1*$^{flox/+}$; *Ctnnb1*$^{lox(ex3)}$ mice, five of which died before necropsy; and *Dhh-Cre*; *Nf1*$^{flox/flox}$; *Ctnnb1*$^{lox(ex3)}$, two of which died before necropsy. *Ctnnb1*$^{lox(ex3)}$ indicates that exon 3 is flanked by *LoxP* sequences in one or both alleles of β-catenin.

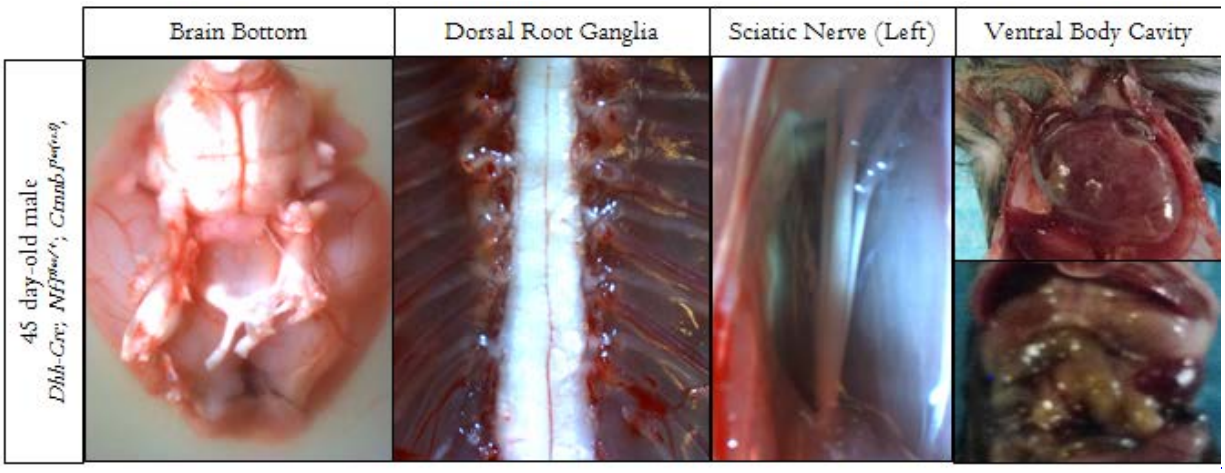

Figure II

Mice were sacrificed using CO_2-euthanasia immediately prior to necropsy. Mice expressing activated β-catenin in Schwann cells showed no significant nerve hyperplasia, as indicated by trigeminal nerves (on bottom of brain), dorsal root ganglia, and sciatic nerves of comparable size and morphology to those of wild-type mice. However, *Dhh-Cre*; *Nf1*$^{flox/+}$; *Ctnnb1*$^{lox(ex3)}$ and *Dhh-Cre*; *Nf1*$^{flox/flox}$; *Ctnnb1*$^{lox(ex3)}$ mice often displayed an enlarged heart and discolored intestines and/or lungs.

Table I

Cohorts of mice generated as of 2/24/14 for tamoxifen inducible *PLP-Cre* mouse model. Breeder mice without *PLP-Cre* allele are not included in this census. 5' GCGGTCTGGCAGTAAAAACTATC 3' and 5' GTGAAACAGCATTGCTGTCACTT 3' used for PCR genotyping of *PLP-Cre* allele.

	PLP-Cre; Ctnnb1$^{lox(ex3)/+}$	*PLP-Cre; Nf1*$^{flox/+}$	*PLP-Cre; Nf1*$^{flox/+}$*; Ctnnb1*$^{lox(ex3)/+}$	*PLP-Cre; Nf1*$^{flox/flox}$*; Ctnnb1*$^{lox(ex3)/+}$
Age (days) as of 2/24/14	85	61	61	32
	85	61	61	
	64	61	55	
	55	61	55	
	39		49	
			49	
			49	
			49	
			39	
			32	
			32	
Total	5	4	11	1

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