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A speedy version of the *double-time* story

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With the recent Nobel prize awarded to Jeff Hall, Michael Rosbash and Mike Young, we were invited to retell the story of *double-time* (*dbt*), a key component of the circadian clock that was identified in the Young lab in the 1990s [1,2]. Hopefully, it will become clear that Nobel-prize winning science is a mixture of bold vision, persistence and lucky breaks.

Circadian (~24hr) rhythms had been studied in the 1950s and '60s as a fascinating phenomenon. However, it was not until Seymour Benzer and his lab bravely attempted to identify clock mutants that the molecular analysis of circadian rhythms opened up, culminating in the 2017 Nobel prize. Ron Konopka, a PhD student in Benzer's lab, identified the first mutants that altered circadian behavior in a forward genetic screen [3]. All three mutations mapped to the same chromosomal

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location and Konopka and Benzer called the affected gene *period* (abbreviated to *per*) since the mutations had such dramatic effects on the period length of fly rhythms. The per^{short} (per^{s}) and per^{long} (per^{l}) mutants changed the fly's normal 24hr rhythms to 19hr and 28hr respectively, while the per^{0} null mutant abolished behavioral rhythms altogether. These mutants were also important for the field of behavioral genetics as a whole since they demonstrated that animal behavior could be profoundly affected by a single gene.

The *period* gene was cloned thirteen years later in the Hall and Rosbash labs at Brandeis University and independently in the Young lab at Rockefeller University [4,5]. Both groups used a new technology: *per* was cloned by transforming per^{0} null mutant flies with P element transposons containing DNA that restored behavioral rhythms, making *per* the first animal gene identified in this way.

However, the sequence of *per* gave no clues for how it functioned in the fly's internal clock. It also seemed highly unlikely that *per* was working alone. So in the late 1980s, Mike Young's lab re-embraced the genetic approach to rhythms and started using P elements to screen for new clock mutants. When P elements insert into the genome, they often disrupt gene function and the P element then marks the affected gene by its position in the genome. In theory, this makes it much easier to identify the affected gene than trying to find a single base pair change caused by chemical mutagens such as EMS that Konopka and Benzer had used to isolate the original *per* mutants.

The P element screen was a success: 23 years after the *per* mutants were published, Amita Sehgal and Jeff Price, two postdocs in Mike Young's lab, described a null mutation in a new clock gene – *timeless* (*tim*) – that made flies arrhythmic just like the original per^{0} null mutation [6]. However, in cloning the gene, Mike Myers, another Young lab postdoc, found that the P element that caused the arrhythmic phenotype was no longer inserted in the *tim* locus but had deleted 70bp of *tim* as it jumped to its final location further along the second chromosome [7]. Thus, although the P element tagging approach did not work as intended, the induced mutation was sufficiently large to identify *tim*.

per and *tim* null mutations do not affect the viability of flies. This facilitated the experiments that led to the discovery of the negative feedback loop that is found at the heart of all circadian clocks – even though clock genes are quite different between animals, fungi and plants. In flies, *per* and *tim* are transcribed, translated and then Per and Tim proteins enter the nucleus, where they repress further transcription of *per* and *tim* [8,9]. Thus *per* and *tim* are components of a molecular clock that gives 24hr rhythms in the levels of *per* and *tim* RNA and proteins.

This was the state of our understanding of the clock in 1996, but two major questions remained unanswered: What activates *per* and *tim* transcription? And how does one cycle of such a simple feedback loop take 24hr to complete? Encouraged by their success in identifying *tim*, the Young lab continued to screen the second chromosome for new clock mutants. However, they reverted to using EMS as a mutagen since the P element screen had not made it particularly easy to identify *tim* and also since EMS can generate point mutations that alter period length – like the original per^{s} and per^{l} alleles. A new postdoc, Jeff Price, a PhD student, Adrian Rothenfluh, and two technicians, Amy Kiger and Marla Abodeely, mutagenized *cn bw* flies, made them homozygous and screened them for altered circadian behavior. Jeff soon found a new mutation with a short period of 21hr. He named the mutant *Speedy* after the cartoon character Speedy Gonzales, although Mike later renamed it $double\text{-}time^{Short}$ (dbt^{S}). Mike was immediately excited by the dbt^{S} phenotype, since a fast-running clock is unlikely to be caused by a non-specific defect and Jeff proceeded to map the mutation.

To everyone's surprise, dbt^{S} mapped to the third

chromosome even though the screen was designed to isolate homozygous second chromosome mutations. Jeff was able to isolate homozygous *dbt*^{S} flies, which had an even shorter period of 18hr. Jeff and a new postdoc in the lab, Brian Kloss, then homed in on the genetic locus first using deletions of the third chromosome and then using P element-containing lines in the affected region. Ultimately, they found one P element (*dbt*^{P}), which failed to complement *dbt*^{S} and behaved like a null allele. This time, the P element proved invaluable in identifying the gene it had inserted into. Interestingly, homozygous *dbt*^{P} animals did not survive to adulthood, indicating that *dbt*^{P} was a lethal mutation and that *dbt* had additional functions outside the clock [2].

Meanwhile Adrian continued to screen for new mutations. Encouraged by the impressive phenotype of *dbt*^{S} heterozygotes, Mike and Adrian decided that screening single flies in an F1 dominant screen would be a more efficient way to identify mutants. This made sense since all of the period-altering mutations identified so far were semi-dominant, meaning their phenotype could be detected even as heterozygotes. And the precision and robustness of the automated locomotor activity assay – and of the flies' internal clock – meant that it was easy to reproducibly detect a fly with less than a 5% change in its period length. Sure enough, Adrian found many new mutants including a new long-period *dbt* allele (*dbt*^{L}) with a heterozygous period of 25hr and a homozygous period of 27hr [2].

Meanwhile a new postdoc, Justin Blau, had joined the Young lab. Mike suggested that Justin study a set of genes that potentially interacted with Per protein, which had been identified in a yeast two-hybrid screen performed in Chuck Weitz's lab at Harvard [10]. However, it was unclear whether these genes were expressed in the same cells as Per *in vivo* or whether their *in vitro* interaction was an artifact of forcing them together in yeast. Justin started by asking where these genes were expressed in *Drosophila* embryos, which the Young lab had extensive expertise with from their studies of *Notch* [11]. Justin focused on late stage embryos since that is when *per* and *tim* are expressed in embryogenesis [12]. However, Justin's somewhat sloppy collections of late embryos included a few animals that had already hatched into larvae. Strikingly, Justin could see *tim* RNA and protein in a few cells in the center of the larval brain. Mike immediately realized that if the molecular clock was functional in larvae – as his earlier work with Amita Sehgal and Jeff Price had suggested [13] – then Justin should be able to study how the clock is affected in *dbt*^{P} homozygous larvae before they died as pupae. Justin found that *dbt*^{P} mutants massively over-accumulate Per protein, indicating that Dbt's normal function is to destabilize Per. Furthermore, rhythms of Per and Tim proteins stopped in the pacemaker neurons in the central brain in *dbt*^{P} larvae, allowing us to conclude that *dbt* is essential for the molecular clock to run [2].

The data from *dbt*^{P} larvae complemented the careful and painstaking time courses of *per* and *tim* RNA and proteins that Adrian performed in *dbt*^{S} and *dbt*^{L} adult flies. The great advantage of studying adult flies is the abundance of *per* and *tim* RNA and proteins in the fly eye, which permits biochemical assays. Adrian found that Per protein accumulation and degradation cycles were altered in *dbt*^{S} and *dbt*^{L} flies – and so too were Per's rhythmic phosphorylation cycles [2]. Alterations to Per phosphorylation made even more sense when Brian used the P element in *dbt*^{P} to identify the affected gene and found that *dbt* encoded the fly version of Casein Kinase Iε – finally, a fly clock gene with a known function and an obvious mammalian counterpart [1]. Importantly, Brian identified amino acid substitutions in *dbt*^{S} and *dbt*^{L} mutants as well as major reductions in *dbt* expression levels in *dbt*^{P} mutants. And Lino Saez, a long term researcher in the Young lab, demonstrated that Dbt stably associates with Per. Together, these data showed that Dbt regulates several steps in the clock and helps extend the simple negative feedback loop to last 24hr [1].

Despite early difficulties in finding mammalian

orthologs of the fly *per* gene, CK1ε and its close relative CK1δ were obvious orthologs of *dbt*. Not surprisingly, Mike was very excited to learn that the *tau* mutation in Syrian hamsters that shortens their behavioral rhythms to 20hr [14] is a missense mutation in CK1ε [15]. Similarly, mutations in human CK1δ alter period length and cause familial advance sleep phase syndrome [16]. Indeed, CK1δ and CK1ε phosphorylate mammalian Per proteins and thus perform a similar function to Dbt in flies. Thus the bold behavioral genetic approach in *Drosophila* initiated by Konopka and Benzer [3], and continued by the Hall, Rosbash and Young labs, has not only shed light on an interesting biological phenomenon in flies, but has also led to an in-depth understanding of mammalian behavior and physiology and even human dysfunction.

The late 1990s were a very exciting time to be in the Young lab as pieces of the clock started appearing ever more rapidly and were fitted together into a coherent whole. In the Young lab, we were not only driven by our own excitement but also by rumors that the Rosbash lab had also identified mutations in new clock genes on the third chromosome. In the end, the Rosbash and Hall labs focused on the *Clock* and *cycle* mutations that encoded the missing transcriptional activators of *per* and *tim* [17,18]. And when we met members of the Rosbash lab at conferences, we realized that the "evil empire" was in reality a friendly group of postdocs and PhD students like ourselves who were easy to get along with.

Although Jeff Hall, Michael Rosbash and Mike Young all have very different personalities, one commonality is that they all remained at the same university for their entire careers as Professors. Such loyalty and such unwavering institutional support is unusual in modern academia and meant that the three prizewinners never had to rebuild a lab from scratch and could stay focused on their research. Additionally, all three winners also had other research interests: Mike Young's lab also studied *Notch*, conveniently located near *per* on the X chromosome; Michael Rosbash's lab also studied RNA processing and Jeff Hall's lab studied *Drosophila* courtship. Perhaps this diversity of interests allowed them all to sustain their long-term excitement for circadian rhythms.

For us lab members, the Young lab was a stable and stimulating environment with an exciting biological question at its heart and with just enough competition to drive us forward. Despite this competition, Mike always gave us the freedom to explore our own ideas and he was never forceful in insisting on a particular research avenue – instead he presented his ideas in such an appealing way that it was hard to disagree! Certainly, Mike's vision, instincts and persistence paved the way for his lab's seminal contributions to the field. We never imagined that we might be doing Nobel prize-winning work and were just excited to figure out how the clock ticks in the tiny fruitfly brain. The same is true of Mike, who was always excited by the latest discoveries in the lab and is a genuinely humble Nobel prizewinner. This was echoed by Rick Lifton, the President of Rockefeller University, who said on the day of the Nobel prize announcement, "The universal comment in my Inbox this morning has been: *I don't know if the Nobel prize has ever been given to a nicer person*." To win the Nobel prize and maintain such modesty is a lesson for us all.

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Mike Young の「狡兎三窟」

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Mike Young の研究室（ロックフェラー大学）に留学していた縁でこの記事の執筆依頼を頂いた。まずは御依頼に従って Mike の人柄を紹介したい。

Mike は常に紳士的で普段からポーカーフェイス。自分からはあまりジョークを言ったりせず、どちらかといえば物静かな人物である。講演も淡々としており、私信のメールでさえビジネスライク。必要な時に必要な事項のみ。この点で、難解な単語が連発する Jeff Hall の長文（Jeff が論文の査読をするとすぐバレる、と評判）や、好き嫌いの感情むき出しの Michael Rosbash の手紙（ハエのリズム屋なら、査読の批判内容でだいたい彼と判る）とは対照的といえる。脱線ついでに。私の中では、ノーベル賞の 3 氏のイメージは日本の 3 大戦国武将と重なる。革新的で好戦的な Rosbash は信長。喜怒哀楽が激しく人情家で変わり者の Jeff は秀吉。ポーカーフェイスで戦略的な Mike は家康… みなさんはどんな印象でしょうか？（受賞講演は YouTube で。3 氏ともいつもより緊張している感じですが）

そんな Mike に今回の受賞のお祝いメールを送っ

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